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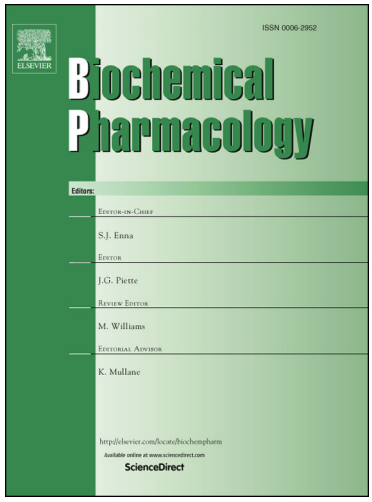

Review

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Behrouz Hassannia, Emilie Logie, Peter Vandenabeele, Tom Vanden Berghe, Wim Vanden Berghe

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Withaferin A: from Ayurvedic folk medicine to preclinical anti-cancer drug

Behrouz Hassannia[1,2], Emilie Logie[3], Peter Vandenabeele[1,2,4], Tom Vanden Berghe[1,2,5], Wim Vanden Berghe[3*]

[1]VIB Center for Inflammation Research; Ghent, Belgium.
[2]Department of Biomedical Molecular Biology; Ghent University, Ghent, Belgium.
[3]Lab Protein Chemistry, Proteomics & Epigenetic Signalling (PPES), Department Biomedical Sciences; University of Antwerp, Wilrijk, Belgium.
[4]Methusalem program; Ghent University, Ghent, Belgium.
[5]Laboratory of Pathophysiology, Department of Biomedical Sciences, University of Antwerp, Wilrijk, Belgium.
*Correspondence: Wim.vandenberghe@uantwerpen.be.

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Abstract

Despite the recent successes of targeted cancer immuno-therapies, drug resistance and disease relapse remain a huge burden in cancer patient treatment. This has fueled renewed interest in natural product discovery to identify new pharmacophores for innovative cancer drug development. Reverse pharmacology approaches of *Withania somnifera* leaves and roots (alternatively also called Ashwagandha or Indian ginseng in traditional Ayurvedic and Unani folk medicine) have identified Withaferin A (WA) as the most bioactive compound for treatment of inflammatory ailments, supporting traditional use of their corresponding extracts in indigenous medicine. In this review we summarize preclinical *in vivo* evidence for therapeutic cancer applications of WA and provide a biochemical framework of its polypharmaceutical effects against cancer hallmarks.

1. Natural products as a source of novel drug discovery

Historically, natural compounds derived from plants have been a rich source for medicinal drug discoveries and have provided leads for anti-cancer agents entering clinical trials [1]. In 1800s, the development of the first commercial drugs based on natural products, such as morphine and aspirin, suggested the birth of a new age in medicine highlighting the capability of successful purification and synthesis of drugs from plants [2]. Today, around half of the FDA-approved anti-cancer small molecules are either natural products or direct derivates from natural products, of which plants are the most compelling source [3]. The vinca alkaloids, vinblastine and vincristine and paclitaxel (Taxol) are among the most well-known plant-derived compounds in the clinic. The natural product epipodophyllotoxin has been a lead for synthesis of clinical anti-cancer drugs etoposide and teniposide [4, 5].

Despite the extensive use of natural products in drug discovery, it is thought that there are still many unexplored plant-based sources used in traditional medicine that could be investigated for new pharmaceuticals in modern medicine. The importance of this latter field can be demonstrated by the antimalarial drug artemisinin derived from the plant *Artemisia annua* (Quinhaosu), which is already known for its long history of use in treatment of fevers in Chinese traditional medicine. Another important drug isolated from traditional medicinal plant is reserpine, the anti-hypertensive agent, which is administered in the Indian traditional medicine as a remedy for snakebite [6]. The genus *Withania* of the Solanaceae (nightshade) family has been used in the indigenous medicine for over the last 3000 years in South east/Southwest Asia, e.g. in the Unani and Ayurvedic systems [7]. The twenty-three known *Withania* species are widely distributed in the drier parts of tropical and subtropical zones, ranging from the Canary Islands, the Mediterranean region and northern Africa to South east/Southwest Asia. In view of its varied therapeutic potential, it has also been the subject of considerable modern scientific attention and appears in WHO monographs on Selected Medicinal Plants. Ashwagandha roots are a constituent of over 200 formulations in Ayruvedha, Siddha and Unani medicine (reviewed in [7]). Today, the growing advances in screening and functional assays could further facilitate exploiting medicinal plants for drug discovery [1, 8].

2. Withaferin A: a promising anti-cancer steroidal lactone isolated from *Withania Somnifera*

Ayurveda, the Indian traditional medicine, is a goldmine for identification of new anti-cancer bioactives in herbal medicinal plants [9, 10]. *Withania somnifera*, also known as Ashwagandha, Indian Winter cherry or Indian Ginseng, is one of the most popular herbs commonly utilized to promote both physical and mental health. Traditionally, the berries and leaves of this plant are applied as a topical remedy for ulcers and tumors. In addition, several reports have linked the health benefits of Ashwagandha to its anti-stress, anti-inflammatory, anti-oxidant, anti-depressant and adaptogenic properties [11-13].

The major chemical constituents of the *Withania* genus, the withanolides, are a group of naturally occurring C28-steroidal lactone triterpenoids built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring [14]. Apart from *Withania*, withanolides are also distributed to other *Solanaceous* genera like *Iochroma, Acnistus, Deprea, Datura, Lycium, Dunalis, Nicandra, Jaborosa, Physalis, Salpichroa, Tubocapsicum, Discopodium, Trechonaetes, and Witheringia*. Moreover, their distribution is not restricted completely to *Solanaceous* plants, withanolides have been reported to be isolated from Taccaceae, Leguminosae, Dioscoreaceae, Myrtaceae, and Lamiaceae families including reports of isolation from marine organisms such as the soft coral *Paraminabea acronocephala* [15].

Reverse pharmacology approaches aiming to identify major bioactive components of Ashwagandha has identified that a highly oxygenated lactone, coined withaferin A (WA) is the most bioactive withanolide responsible for a variety of health beneficial effects of Ashwagandha [10, 11] (Figure 1). A growing list of reports have shown that WA exhibits anti-inflammatory, anti-diabetic, anti-cancer and anti-angiogenesis effects [13, 16], which has fueled worldwide interest to resolve its mechanism(s) of action. Structural analysis of WA has revealed 3 positions, including the unsaturated A-ring at C3, the epoxide structure at position 5 and C24 in its E-ring, as the most potential reactive sites susceptible for nucleophilic attack (Figure 1). It has been demonstrated that these sites can covalently bind to cysteine residues of proteins through Michael addition alkylation reactions, thereby causing loss of activity of the target protein [13]. Importantly, among the different positions, the C27 hydroxyl group is not necessary for biological activities of WA and can be conjugated with biotin creating biotinylated WA to identify WA target proteins [17, 18].

3. Pharmacokinetic profile of WA

Pharmacokinetic studies in mice have shown that WA has a rapid oral absorption and reaches to peak plasma concentration of around 16.69 ± 4.02 ng/ml within 10 minutes after oral administration of *Withania somnifera* aqueous extract at dose of 1000 mg/kg, which is equivalent to 0.458 mg/kg of WA [19]. Following an intraperitoneal injection of WA in mice at a single 4 mg/kg dose, WA reached a maximum plasma concentration up to 2 μM in with a half-life of around 1.4 hour. This shows that systemic achievable WA concentrations correspond with a pharmacologically therapeutic effective window (nanomolar to low micromolar range). The clearance of WA from plasma was rapid and WA was undetectable in mice after 24 hours [20]. Moreover, one month intraperitoneal WA treatment (4mg/kg) in a breast cancer metastasis mouse model, showed dose-dependent inhibition of metastatic lung nodules with limited adverse toxicity, as evaluated by presence of fibrosis and/or necrosis of the pulmonary parenchyma [20]. Occasional reports on weight loss effects upon chronic WA treatment have recently been explained by WA specific sensitization of leptin receptor signaling [16, 20, 21]. Furthermore, a toxicity study in rats identified no-observed-adverse-effects after oral administration of *Withania somnifera* extract with 3% WA at a dose of 2000 mg/kg [22]. Recently, the safety and pharmacokinetics of root extract of *Withania somnifera,* containing 4.5% of WA w/w, was evaluated in a phase I trial in patients with advanced stage high-grade osteosarcoma. Up to 4800 mg extract, equivalent to 216 mg of WA per day, was well tolerated in patients without any dose limiting toxicity, revealing a good safety profile for oral administration applications of WA [23].

4. Anti-cancer activities of WA

Today, anti-cancer activities of WA have been documented in variety of cancers cells such as glioblastoma, neuroblastoma, multiple myeloma, leukemia, breast, colon, ovarian and head and neck cancer [21, 24, 25]. Accumulating reports have corroborated the tumor growth inhibition effect of WA in diverse mouse cancer models (Table 1). In addition, the combination treatment of WA with several therapeutic agents or modalities have been shown to improve efficacy of standard chemotherapy or to overcome drug resistance (Table 2).

The molecular mechanism underlying the anti-tumor activity of WA is not completely understood, however, it seems to involve polypharmaceutical effects such as targeting

cytoskeleton structure and proteasomal system, regulating heat shock proteins activity, reactive oxygen species (ROS)-mediated cytotoxicity, inhibition of nuclear factor kappa B (NF-κB) and oncogenic pathways (Figure 2) [13, 26, 27].

5. Biochemical framework for anti-cancer actions of WA

A broad range of molecular biochemical targets and biological effects of WA have been described (Figure 2), which apparently depend on the applied concentration range of WA, context and genetic makeup of the cells.

5.1. Proteasomal inhibition

Malignant cells are known to synthesize larger quantities of proteins compared to their healthy counterparts. In order to prevent the (toxic) accumulation of unneeded or misfolded proteins, a system needs to be in place that can monitor and resolve the protein excess that occurs in the cells. The ubiquitin proteasome system, the major pathway for protein degradation in eukaryotic cells, is able to hydrolyze ubiquitin-tagged target proteins and thus reduce the risk for proteotoxicity. In addition, the proteasome is involved in the regulation of many crucial processes in cancer cells such as NF-κB activation, cell cycle regulation, p53 activity and endoplasmic reticulum (ER) stress, making it an attractive therapeutic target. Indeed, tumor cells are known to be more prone to proteasome inhibition than normal cells and there are already a few drugs on the market that make use of this mechanism, especially in the context of multiple myeloma [28-30].

Accumulation of ubiquitinated proteins after WA treatment in different cell lines has suggested that WA is a potent inhibitor of the ubiquitin-mediated proteasome pathway. Micromolar concentrations of WA (10 μM) could suppress the chymotrypsin-like activity of the proteasome system in both prostate cancer and malignant pleural mesothelioma cells, which was reflected in the accumulation of ubiquitinated proteins and proteasome target proteins, Bcl-2-associated X protein (Bax), p27 and IκBα [31, 32]. Alternatively, the PSMB10 proteasome subunit was identified as a direct WA target for proteasome inhibition [18]. However, in a contradictory report, WA was shown to induce degradation of HSP90 target proteins in pancreatic cancer cells, which can be blocked by the proteasome inhibitor MG132 [33]. These finding suggests that inhibitory effects of WA on the proteasome complex might be cell context-related or is dependent on the concentration of WA.

5.2. Cell cycle inhibition

Micromolar concentrations of WA (1-3 μM) have been reported to inhibit cell proliferation by inducing G2 and M phase cycle arrest in ovarian, breast, prostate, gastric and myelodysplastic/leukemic cancer cells and osteosarcoma [34-39]. This inhibition is modulated by different mechanisms. Firstly, WA is able to decrease the cyclin-dependent kinase 1 (Cdk1) activity and prevent Cdk1/cyclin B1 complex formation, which are key steps in cell cycle progression [34, 40]. By depleting the levels of the cell division cycle 25 (cdc25) phosphatase proteins, the inhibitory threonine phosphorylations on Cdk1 are no longer removed and result in cell cycle arrest after WA treatment [34, 35, 38, 40]. Secondly, studies in colorectal adenocarcinoma cells demonstrated that WA causes mitotic delay by inducing the degradation of mitotic arrest deficient 2 (Mad2), a spindle assembly checkpoint (SAC) protein, and its interacting partner cdc20 [41].

5.3. Oxidative stress modulation

Increasing ROS and oxidative stress is considered an effective anti-cancer therapy. Generally, cancer cells have higher levels of ROS than normal cells, which is thought to render them more sensitive to agents that promote further accumulation of ROS [42]. Several chemotherapeutics currently used in cancer therapy, such taxanes, vinca alkaloids and anthracyclines, effectively trigger high oxidative stress resulting in cell death [42, 43]. Micromolar concentrations of WA (2-6 μM) also modify the redox potential and enhances oxidative stress in various types of cancer cells [44-46]. This elevated oxidative stress triggers cell death in different malignancies including leukemia, breast cancer, melanoma and renal cells [35, 40, 44, 45]. The inhibition of the mitochondrial complex III activity has been shown as a mechanism of WA-induction of ROS in breast cancer cells [46, 47]. We recently identified that micromolar concentrations of WA (1-10μM), in addition to an oxidative burst, induces lipid peroxidation in neuroblastoma which promotes ferroptotic cell death [21, 48].

However, extracts of *Withania somnifera* showed anti-oxidant activity as well. WA treatment (40 mg/kg) reduces acetaminophen-induced liver injury (AILI) in mouse models and decreases H_2O_2-induced glutathione (GSH) depletion and necrosis in hepatocytes [49]. The analysis of this protection mechanism points out that WA triggers an anti-oxidant response after acetaminophen overdose by enhancing hepatic transcription of the nuclear factor erythroid

2–related factor 2 (NRF2)-responsive genes [49-51]. Through direct interaction of WA with kelch-like ECH-associated protein 1 (KEAP1), NRF2 ubiquitination and consequent proteasomal degradation is prevented, resulting in an increased expression of various detoxification and anti-oxidant proteins [51-54]. This mode of action of WA can be observed in different cell types, including neuronal cells, suggesting that WA could benefit diseases which are linked to aberrant cellular stress [51, 55]. Depending on the dose of WA, NRF2 activation precedes protein folding, heat shock and ubiquitination stress responses aiming to restore proteostasis, or triggering cell death upon excessive proteotoxic stress and massive ROS production [51, 56-59].

5.4. Inhibition of Wnt dependent cancer hallmarks

Mutations in the Wnt signaling pathway are recognized to be one of the critical causes for carcinogenesis, since Wnt regulates multiple cancer hallmarks, such as metastasis and immune evasion [60-63]. In most cases, hyperactivation of the Wnt pathway triggers normal cells towards malignant transformation [64, 65]. Interestingly, micromolar concentrations of the WA analogue 3-azido WA (0.75-3μM) can indirectly inhibit Wnt signaling and prevent cancer transformation [66]. In androgen-refractory prostate cancer cells, WA and 3-azido-WA upregulate the pro-apoptotic prostate cancer response-4 (PAR-4) protein [66, 67]. This interferes with Wnt signaling through modulation of β-catenin phosphorylation [66]. In particular, high concentrations of PAR-4 suppress Akt kinase activity which results in activation of glycogen synthase kinase 3 beta (GSKβ). This latter kinase phosphorylates β-catenin known to block the canonical Wnt signaling pathway [66].

5.5. Inhibition of EMT and uPA dependent cancer invasion and metastasis

WA can also block tumor metastasis through reduced expression of epithelial mesenchymal transition (EMT) markers. Typically, EMT promotes aggressive cell proliferation, marked with a high invasion and metastasis capacity. By upregulating EMT promoting transcription factors like Slug (SNAI2) and Twist, expression of the adhesion molecule E-cadherin is significantly reduced thereby allowing for an increased cellular mobility [68]. Several studies have now shown that WA can counter the EMT by downregulating these critical transcription factors and restoring E-cadherin expression [69, 70]. This effect has also been observed in breast cancer xenograft and MMTV-neu mice models, where next to increased E-cadherin

expression an additional decrease in vimentin, a mesenchymal marker regulating cell motility, was observed [71]. Another finding indicates that 3-azido-WA upregulates the expression of E-cadherin and TIMP-1 in prostate cancer cells [66]. Furthermore, nanomolar concentrations WA (700nM) exert anti-metastatic activities in breast cancer cells through inhibition of the urokinase-type plasminogen activator (uPA) protease [72]. Notably, the uPA system participates in remodeling of the extracellular matrix and promotes cell proliferation and migration. In addition, high expression of uPA is associated with breast cancer relapse and metastasis [72, 73].

5.6. Induction of cancer stem cell senescence

Recent studies have reported that WA can also interfere with two other crucial cancer hallmarks: evasion of cellular senescence and maintenance of the tumor neoplasm via cancer stem cells. Typically, when a cell is exposed to oncogenic stressors, a signal transduction pathway is initiated that restricts proliferation of the damaged cells by triggering irreversible cell cycle arrest [74, 75]. Naturally, this is a crucial tumor suppression mechanism which, unfortunately, most cancer cells are able to bypass. Additionally, the malignant cells can sustain themselves through a small population of long-lived stem cells, which have the capacity to self-renew [76, 77]. To eradicate the tumor, it is thus vital that these cancer stem cells (CSCs) are targeted as well. However, due to their high expression of repair enzymes, anti-apoptotic proteins and efflux transporters, they are often resistant to classical cancer therapy including chemotherapy [78].

WA treatment has multiple inhibitory effects on CSCs of different cancer types. Multiple myeloma cancer stem cells were found more sensitive than normal hematopoietic stem cells to WA treatment [79]. Nanomolar concentrations WA (125-500 nM) suppress tumor sphere formation indicating that the self-renewal of CSC is abolished [70]. This can even be observed in primary spheroids, isolated from MMTV-neu mice [80]. Interestingly, the use of combination therapies, such as cisplatin/WA or DOXIL/WA, can further enhance the inhibition of spheroid formation in ovarian CSC while neither cisplatin nor DOXIL are able to affect the CSCs on their own [81, 82]. Next to self-renewal, invasion and mobility capacity are affected by WA exposure as well [70, 83]. The loss of these CSC-specific characteristics is reflected in the loss of typical stem cell markers such as ALDH1A, Nanog, Sox2, CD44 and CD24 [70, 79-83]. Overall, WA treatment seems to direct the CSCs toward more differentiated tumor cells

whereby sometimes even a senescent state is acquired [70, 84]. Induced CSCs, created from human MCF-10A cells, showed an increased senescent character after incubation with WA, marked by a drastic change in cell morphology [70]. Moreover, senescence associated markers such as β- galactosidase and γH2AX were both present in these differentiated cells, indicating that the CSC properties are indeed inhibited by WA-induced senescence [70]. The underlying mechanism of this phenomenon is not completely understood although two independent studies have identified p21, a cyclin-dependent kinase inhibitor, as a crucial player in WA-induced senescence [70, 84].

5.7. Inhibition of glycolysis and tricarboxylic metabolism

During carcinogenesis, malignant cells are obliged to reshape their energy metabolism to allow the increasing proliferation rate that is characteristic for nearly all tumors. One of the most drastic changes that takes place, is a shift in anabolic metabolism from oxidative phosphorylation to glycolysis, also known as the Warburg effect [85]. This increased glycolysis grants the cancer cells many survival advantages such as acidification of extracellular environment due to lactate production, which reduces the viability of surrounding normal cells and promotes the activation of metalloproteinases that are crucial for tumor invasion [86]. Intraperitoneal administration of WA (4mg/kg) exerts anti-tumor effects in MMTV-neu mice by suppressing the glycolysis and tricarboxylic (TCA) cycle [87]. Treatment of mammary cancer with WA leads to significant alterations in concentrations of various biochemicals, where most correlated to a decrease in glycolysis and TCA cycle [87]. Importantly, the tumors of WA-treated mice were found to have a highly consistent drop in lactate levels compared to control mice. In addition, WA can decrease the levels of isocitrate dehydrogenase (IDH), which has been identified as a potent oncogene [88, 89]. The changes in TCA enzymes and intermediates have also been demonstrated in other *in vivo* cancer models [90].

5.8. Reversing cancer epigenetic hallmarks

Besides genetic mutations, epigenetic changes in DNA methylation patterns at CpG sites (epimutations) or deregulated chromatin states of tumor promoting genes and noncoding RNAs has emerged as novel governing factors in tumor progression and cancer drug sensitivity [91]. Since epigenetic marks (epimutations) are reversible in contrast to genetic defects and several plant secondary metabolites have reported epigenetic effects, WA has also been evaluated for its ability to reverse adverse epigenetic marks in cancer (stem) cells

or to attenuate tumorigenesis-progression, prevent metastasis or sensitize for drug sensitivity [92, 93]. Remarkably, we and others found that WA triggers multiple epigenetic changes involved in suppression of different cancer hallmarks associated with cell cycle regulation, cell death, cancer cell metabolism, cell motility and metastasis [72, 94, 95]. Moreover, nanomolar concentrations WA (700 nM) reprogram the epigenome of drug resistant triple negative breast cancer cells towards therapy sensitive luminal type breast cancer cells, by triggering gene selective DNA hypermethylation and chromatin silencing (i.e. PLAU, ADAM8, TNSF12, ME3), through regulation of DNA Methyltransferase 1 (DNMT), histone lysine demethylases jumonji KDM and histone deacetylase (HDAC) family chromatin writer-eraser-enzymes [72, 94, 96]. In multiple myeloma, HDAC6 was identified as a direct WA target [18].

5.9. Induction of cell death

Micromolar concentrations of WA (1-10μM) are known to induce selective cytotoxicity in various types of tumor cells through different pathways, as illustrated in Figure 3. WA can change the activity of multiple kinases such as mitogen-activated protein (MAP) kinase p38, c-Jun N-terminal kinase (JNK), AKT and extracellular signal-regulated kinase (ERK), ribosomal S6 kinase (RSK) and inhibitor of nuclear factor kappa-B kinase β (IKKβ) [97-102]. In leukemia cells, WA-triggered apoptosis is associated with activation of MAP kinase p38 signaling cascade, downregulation of BCL2 and upregulation of Bcl-2-associated X (BAX) [101]. WA-mediated apoptosis in breast cancer cells is regulated by Forkhead Box O3a (FOXO3a) and its transcriptional target Bim [103]. Accordingly, knockdown of Bim confers resistance to WA-induced apoptosis [103]. WA-dependent inhibition of Notch-1 and signal transducer and activator of transcription 3 (STAT3) signaling further contribute to cancer cell apoptosis [104-106]. Accordingly, overexpression of inhibitor of apoptosis proteins (e.g. XIAP, cIAP2, and Survivin) in breast cancer cells delays WA-induced cell death [107]. ER stress mediated apoptosis has been attributed to WA cytotoxicity in human renal carcinoma cells. Silencing of C/EBP homologous protein (CHOP) impeded WA-induced apoptosis in these cells [108]. In a T-cell leukemic cell line, WA triggers apoptosis by acting as an agonist of liver X receptor α (LXR-α). LXR-α is important for the regulation of cholesterol, fatty acid, and glucose homeostasis, however its specific role in WA-mediated apoptosis is not clearly defined [109]. In a recent study, we discovered that WA induces a form of non-apoptotic cell death coined ferroptosis in neuroblastoma. In ferroptosis, uncontrolled lipid peroxidation of cellular

membranes leads to membrane damage and finally a necrotic cell death [48, 110]. We identified that WA induces ferroptosis by direct targeting and inactivating the main lipid repair enzyme glutathione peroxidase 4 (GPX4). GPX4 detoxifies toxic lipid hydroperoxides of the membranes, thereby prevents membrane from lipid peroxidation derived cell death [111]. Furthermore, we found that WA elevates the levels of intracellular labile ferrous iron (Fe^{+2}) through excessive activation of heme oxygenase-1 (HMOX1), which independently causes accumulation of toxic lipid radicals and ensuing ferroptosis [21]. Of note, WA-induced ferroptosis resulted in suppressing neuroblastoma xenografts growth and relapse rate [21].

5.10. Induction of antitumor immunity

The tumor microenvironment can exert a profound immunosuppressive effect on effectors T cells by secreting TGF-β and IL-10 which allows the tumor to escape from the patient's immune defense system during progression and growth. WA has potential to fine-tune the immunosuppressive phenotype of tumor-associated lymphocytes by modulating immuno-suppressive cytokines, as shown by selective inhibition of Treg proliferation and induction of apoptosis in Tregs cells in immune competent PCa mouse model treated with WA. Additionally, significant inhibition of tumor growth in immune competent PCa mouse model as compared to immune deficient xenograft suggests an additional role of WA on reversal of anti-tumor immune response from immune suppression to immune competence [112].

Moreover, *in vivo* treatment of tumor-bearing mice with WA (4 mg/kg), decreased tumor weight, reduced the number of granulocytic myeloid-derived suppressor cells (MDSC), and blocked MDSC-mediated suppression of $CD4^+$ and $CD8^+$ T cells activation. These findings suggest that WA may be used as adjunctive cancer immunotherapy to facilitate the anti-tumor immunity [113]. It is interesting to note that a wide variety of cancer immunotherapies are emerging, but many of them suffer from unwanted side effects from overactivation of the adaptive immune response. Since WA combines peripheral immunosuppression wit MDSC specific enhancement of tumor specific T cell activation, WA may be selected as an ideal candidate for further development as a cancer immunotherapy [114].

5.11. Targeting cytoskeletal proteins

The cytoskeleton is composed of actin, the microtubule network and intermediate filaments and remains one of the main targets in cancer treatment. The strategy is based on the interference with the rapid proliferation of the neoplastic cells. Interestingly, mounting

evidence suggests that the cytoskeleton contributes in EMT and cancer metastasis as well [115]. It is proposed that WA exerts some of its anti-cancer and anti-invasiveness effects through perturbation of the cytoskeleton structure of cancer cells.

Using 4 μM biotinylated WA, the human annexin II protein was identified as a direct binding target of WA [116]. WA disrupts the interaction between annexin II and F-actin by covalent binding to Cys133 of human annexin II, which leads to aggregation of actin. Therefore, massive cytoskeleton disruption by WA abrogates invasiveness of cancer cells [116]. The cytoskeleton-perturbing activity of WA has also been linked to the disassembly of vimentin. Vimentin is a member of the type III intermediate filaments which, together with microtubules and actin microfilaments, constitute the cytoskeleton [13]. In addition to structural functions, it is recognized that vimentin can serve as a signaling platform or scaffold for signaling molecules such as kinases, thereby modulating their distribution and activity [13]. The high expression of vimentin in cancer cells and its correlation with EMT and metastasis makes vimentin an attractive therapeutic cancer target [13, 117]. WA was characterized to bind to Cys327 of human vimentin leading to vimentin depolymerization and filament aggregation [118]. In line with this finding, inhibition of breast cancer invasion and metastasis was associated with WA-induced phosphorylation (Ser56) and depolymerization of vimentin [20]. Mechanistically, it was specified that the WA-induced aggregation of vimentin filaments in perinuclear region impedes the formation of lamellipodia required for cell motility [119]. Moreover, anti-angiogenic activity of WA strongly depends on vimentin, since deficiency of vimentin masks inhibition of neovascularization by WA [118, 120].

Beside targeting annexin II and vimentin, WA can additionally interfere with the microtubule network. This network is composed of tubulin heterodimers and is crucial for cell cycle progression, cellular movement, and intracellular trafficking [121]. A recent study in breast cancer cells revealed that treatment with WA results in downregulation of β-tubulin, aberrant microtubule spindle formation and G2-M phase cell cycle arrest. Further analysis revealed that WA binds to Cys303 of human β-tubulin, proposing microtubules as another cytoskeletal target of WA [121].

5.12. Targeting heat shock proteins and chaperones

Heat shock proteins (HSPs), an evolutionary family of proteins acting as molecular chaperones, are overexpressed in variety of human cancers and are important players in tumor cell proliferation, differentiation, invasion, metastasis [122]. In particular, higher levels of HSP90 are vital for cancer cells due to its critical role in proliferation and stress responses. This selective 'HSP90 addiction' in malignant cells has proven to be an effective therapeutic target with several HSP90 inhibitors being currently investigated in clinical evaluation [123]. In this context, it is exciting that micromolar concentrations of WA (10 μM) were found to inhibit the chaperone activity of HSP90 through binding and disruption of the HSP90-Cdc37 complex [33]. This Cdc37 protein is a critical co-chaperone of HSP90, which serves as an adaptor to load the kinase client proteins into HSP90 complex for maturation [124]. Inhibition of HSP90-Cdc37 complex activity, and the subsequent degradation of HSP90 target proteins, has been proposed as a mechanism of action of WA in pancreatic cancer cells and B-cell lymphoma [33, 125].

Next to HSPs, various other chaperone proteins exist in the cell. For instance, p97 is an ATPase associated with various cellular activities (AAA+) type II chaperone that is involved in protein quality control as well as variety of other cellular functions associated with cancer [126, 127]. Notably, upregulation of p97 has been implicated in various types of malignancies and is correlated with a poor clinical outcome [126]. As a prototype function, p97 contributes to the quality control mechanism in the endoplasmic reticulum (ER) through the endoplasmic-reticulum-associated protein degradation (ERAD) process [128]. In ERAD, p97 dislocates the misfolded proteins from ER to cytosol for degradation through the ubiquitin proteasome system [128]. In a recent study, some of WA analogues were found to inhibit the ATPase activity of p97 thereby impairing the quality control machinery [127].

One of the mechanisms by which immunogenic cell death (anti-cancer immune response) occurs is linked to the induction of an unfolded protein response (UPR) due to prolonged chemotherapeutic stress on cancer cells, characterized by cell surface expression of heat shock proteins (HSPs) including HSP70 and HSP90 as well as ER-stress mediated pre-apoptotic expression of the ER chaperone calreticulin (CRT). Furthermore WA was identified as a chemoimmunotherapeutic agent by translocating known immunogenic HSPs HSP70, CRT and gp96 to the cell surface of several cancer cell lines, which upon apoptosis can confer immunological protection against re-challenge with live tumor cells in vivo [129].

5.13. Inhibition of NF-κB kinase signaling pathway

It is known that the transcription factor NF-κB plays a major role in oncogenesis. The constitutive NF-κB signaling in cancers promotes inflammation and enhances cell survival by upregulating anti-apoptotic genes [130]. Inhibition of NF-κB by WA (1μM) alone or in combination with other cancer therapies results in tumor cell death or growth inhibition [131]. The capability of WA to inhibit NF-κB activation has been examined in different cell lines [67, 132-137]. WA was shown to hamper TNF and LPS-induced NF-κB activation by suppressing the degradation of phosphorylated-IκBα [132, 134]. Furthermore, WA can block TNF-induced activation of NF-κB by preventing IKKβ kinase activity through a thioalkylation-sensitive redox mechanism upon targeting Cys179 in the catalytic site of IKKβ [132, 138].

6. Final conclusion and perspectives

Despite its pleiotropic effects *in vitro*, different xenograft tumor models (Table 1) reveal remarkable effective and selective anti-cancer properties of WA *in vivo*. Of special note, therapeutic doses of WA were found to be non-toxic for normal peripheral blood mononuclear cell derived lymphocytes and monocytes [101, 109]. Also, the normal mammary epithelial cell lines HMEC and MCF-10A are more resistant to WA-induced cell death compared to breast cancer cells [44-46, 103]. Along the same line, fibroblasts are less responsive to WA in comparison to cancer cells [13, 139-141]. *In vivo*, the anti-cancer dose of WA seems to trigger minimal toxicity to normal tissues [20]. It must be noted that pharmacokinetic studies in mice indicate a rapid clearance of WA in plasma [20]. Considering the rapid decrease in concentration of WA in plasma, possible protective anti-oxidant responses at low subtoxic concentrations might rescue tumor cells, which includes a risk for further therapeutic clinical cancer applications of WA. Therefore, pharmacokinetic properties of WA metabolites in relation to pro/anti-oxidant responses need to be taken into account for further (pre)clinical development of WA as an anti-cancer drug. Of special interest, in a recent study we could demonstrate complete therapy response in a neuroblastoma xenograft model with WA, whereas tumor relapse was observed upon etoposide treatment few weeks post-treatment [21].

Moreover, since poor oral bioavailability limits the use of many bioactive phytochemicals in the prevention and treatment of cancer, novel coated polymeric polycaprolactone implants have been developed for sustainable release of anti-cancer bioactives. Compounds are dose dependently released from the implants in vitro and for long durations (months), which is correlated with in vivo release. For example, WA given via the implants significantly inhibited human lung cancer A549 xenograft in athymic nude mice, while it was ineffective when the same total dose was administered intraperitoneally and required over 2-fold higher dose to elicit effectiveness [142, 143]. Along the same line, cervical implants, containing 2% and 4% WA, with 8 coats of blank polymer, provided sustained release of WA for a long duration for treatment of HPV induced cervical intraepithelial [144]. Finally, we formulated WA in a novel amphiphilic degradable pH-sensitive nanocarrier, which allowed systemic administration of WA and suppression of tumor growth [21].

In conclusion, WA holds promise as a pleiotropic therapeutic anti-cancer compound *in vitro* as well as *in vivo*, which warrants further (pre)clinical development [145-147]. Considering that cancer is a disease with multiple dysregulated stress signaling pathways, the polypharmaceutical effects of WA can be a therapeutic advantage in cancer treatment. Particularly since single-target drugs easily allow escape pathways or evoke therapy resistance and disease relapse, simultaneous targeting of various cancer hallmarks by WA may improve therapeutic outcome in patients by preventing or overcoming drug resistance [148].

Tables

Table 1. Anti-cancer activities of WA *in mice*.

Cancer	Animal model	Dose	Mechanism	References
Prostate	PC-3 xenograft in nude mice	5 mg/kg	PAR4-dependent apoptosis	[67]
Prostate	PC-3 xenograft in nude mice	4-8 mg/kg	Inhibition of proteasome system	[31]
Breast	MDA-MB-231 xenograft in nude mice	4 mg/kg	FOXO3a and Bim-dependent apoptosis	[103]

Table 1. Anti-cancer activities of WA *in mice*.

Cancer	Animal model	Dose	Mechanism	References
Breast	MDA-MB-231 xenograft in nude mice	4 mg/kg	Activation of ERK/RSK and CHOP/Elk1, upregulation of DR5	[99]
Pancreas	Panc-1 xenografts in nude mice	3.6 mg/kg	HSP90 inhibition and degradation of Akt and Cdk4	[33]
Medullary thyroid cancer	DRO 81-1 xenografts in nude mice	8 mg/kg	Inhibiton of RET proto-oncogen phosphorylation and activation	[149]
Cervial	CaSki xenograft in nude mice	8 mg/kg	Downregulation of HPV16 E6 and E7, increasing p53 protein levels	[40]
Mammary tumor	4T1 spontaneous mouse mammary carcinoma model	4 mg/kg	Phosphorylation and disassembly of vimentin	[20]
Ovarian (WA/doxorubicin)	A2780 xenograft in nude mice	WA 2 mg/kg, Doxorubicin 1 mg/kg	Induction of ROS and autophagy	[150]
Uveal melanoma	92.1 xenograft in SCID mice	8 mg/kg, 12 mg/kg	Inhibition of Akt, inhibition of c-MET activation	[151]
Mammary tumor	MMTV-neu mice	100 μg	Suppression of glycolysis and TCA cycle Inhibition of self-renewal of cancer stem cells	[80, 87]
Ovarian (WA/cisplatin)	A2780 xenograft in nude mice	WA 2 mg/kg, Cisplatin 6 mg/kg	Inhibition of Notch-1, downregulation of cancer stem cells	[83]
Colon	HCT116 xenograft in nude mice	2 mg/kg	Inhibition of STAT3 phosphorylation and activation	[106]
Skin	TPA skin cancer model	20 μg	Suppresstion of AP-1 and, inhibition of transcription of ACC1 gene	[152]

Table 1. Anti-cancer activities of WA *in mice*.

Cancer	Animal model	Dose	Mechanism	References
Pancreas (WA/Oxaliplatin)	Panc-1 xenografts in nude mice	WA 3mg/kg, Oxaliplatin 10 mg/kg	Mitochondrial dysfunction, induction of ROS, inactivation of PI3K/AKT pathway	[97]
Colorectal	HCT-116 xenograft in nude mice	5 mg/kg	Inhibiton of AKT, downregulation of EMT markers	[69]
Ovarian (WA/DOXIL)	A270 intraperitoneal tumors in nude mice	WA 2 mg/kg, DOXIL 2 mg/kg	Targeting cancer stem cells	[82]
Mammary Tumor (3-WA)	4T1 spontaneous/orthotopic mouse mammary carcinoma model	10 mg/kg	Inhibition of Ras-Mnk and PI3-AKT-mTOR, inhibiton of eIF4E phosphorylation and protein translation, downregulation of c-FLIP	[153]
B-cell lymphoma	A20 allograft in Balb/c mice	12 mg/kg	HSP90 inhibition	[125]
T-cell ALL (WA/DBZ)	Notch1-mutant T-ALL xenograft in NRG mice	WA 10 mg/kg, DBZ 10 mg/kg	eIF2A-dependent translation inhibition	[25]
Neuroblastoma	IMR-32 xenograft in nude mice	4 mg/kg	GPX4 inhibition, HMOX1 activation	[21]

PAR4, prostate apoptosis response 4; FOXO3, Forkhead Box O3; ERK, extracellular-signal-regulated kinase; /RSK, ribosomal S6 kinase; CHOP, CCAAT-enhancer-binding protein homologous protein, DR5, death receptor 5; HSP90, heast shock protein 90; cdk4, cyclin-dependent kinases 4; ROS, reactive oxygene species; EMT, epithelial-mesenchymal transition; MET, mesenchymal epithelial transition; STAT3, signal transducer and activator of transcription 3; AP1, activator protein 1; ACC, acetyl-coa carboxylase; 3-WA, an analogue of WA; mTOR, mammalian target of rapamycin; FLIP, FLICE; fas-associated protein with death domain (FADD)-like IL-1β-converting enzyme; DBZ, γ-secretase inhibitors; ALL, Acute lymphoblastic leukemia; GPX4, glutathione peroxidase 4; HMOX1, heme oxygenase 1.

Table 2. Chemosensitisation effects of WA in combination treatment

Compound	Cancer cell	Chemosensitisaton Mechanism (Analytical method synergism)	References
WA+TRAIL	Renal cancer cells	WA sensitizes cells to TRAIL-induced apoptosis by upregulation of DR5 and down regulation of c-FLIP.	[154]

Table 2. Chemosensitisation effects of WA in combination treatment

Compound	Cancer cell	Chemosensitisaton Mechanism (Analytical method synergism)	References
WA + Sorafenib	Papillary and anaplastic cancer cells	Combination treatment enhances cell cycle arrest and apoptosis at lower dose of each drugs. (Synergistic effects were demonstrated via isobologram/combination -index analysis)	[155]
WA + Doxorubicin	Epithelial ovarian cancer cells	WA decreases the dosage required for effects of doxorubicin by induction of ROS and autophagy (Synergistic effects were demonstrated via combination-index /isobologram analysis)	[150]
WA+TRAIL WA + Etoposide WA + Celecoxib	Breast cancer cells	WA enhances TRAIL-, etoposide-, celecoxib-, and TRAIL-induced DR5 expression and results in higher inhibition of clonogenicity	[99]
WA + Cisplatin	Ovarian cancer cells	WA enhances cisplatin-induced cell migration suppression by Notch-1 inhibition and downregulation of cancer stem cell markers (Synergistic effects were demonstrated via combination-index /isobologram analysis)	[83, 156]
WA + Oxaliplatin	Pancreatic cancer cells	WA enhances oxaliplatin-induced growth suppression and apoptosis by inactivation of the PI3K/AKT survival signaling pathway and triggering oxidative stress (Synergistic effects were demonstrated via combination-index /isobologram analysis)	[97]
WA + Doxil® (Doxorubicin Liposomal)	Ovarian cancer cell	WA enhances DOXIL-induced suppression of cell prolifreation by inhibition of ALDH1 and Notch1. Combination therapy resultes in a significant reduction in tumor growth, and complete inhibition of metastasis . (Synergistic effects were demonstrated via combination-index /isobologram analysis)	[82]
WA + TTFields	Glioblastoma cells	WA enhances TTFields-induced cell proliferation inhibition (Synergistic effects	[157]

Table 2. Chemosensitisation effects of WA in combination treatment

Compound	Cancer cell	Chemosensitisaton Mechanism (Analytical method synergism)	References
		were demonstrated via 2-way Anova and Poisson regression analysis)	
WA + DBZ (γ-secretase inhibitors GSIs, Notch1 inhibitors)	T-ALL leukemia cells	Combination of WA and DBZ enhances apoptotic response by inhibition of eIF2A-dependent translation and NOTCH inhibition. Withaferin A enhances antitumor effects of Notch inhibition with DBZ in vivo in a mouse model of Notch1-induced T-ALL (Synergistic effects were demonstrated via combination-index /isobologram analysis)	[25]

TRAIL, TNF-related apoptosis-inducing ligand; DR5, death receptor 5; FLIP, FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; ALDH1, aldehyde dehydrogenase; TTFields, tumor treating fields; GSI, γ-secretase inhibitor.

Figure legends

Figure 1: Structure of WA.

Figure 2: Overview of molecular targets and pathways affected by WA.

Figure 3: Mechanism of WA-induced cell death.

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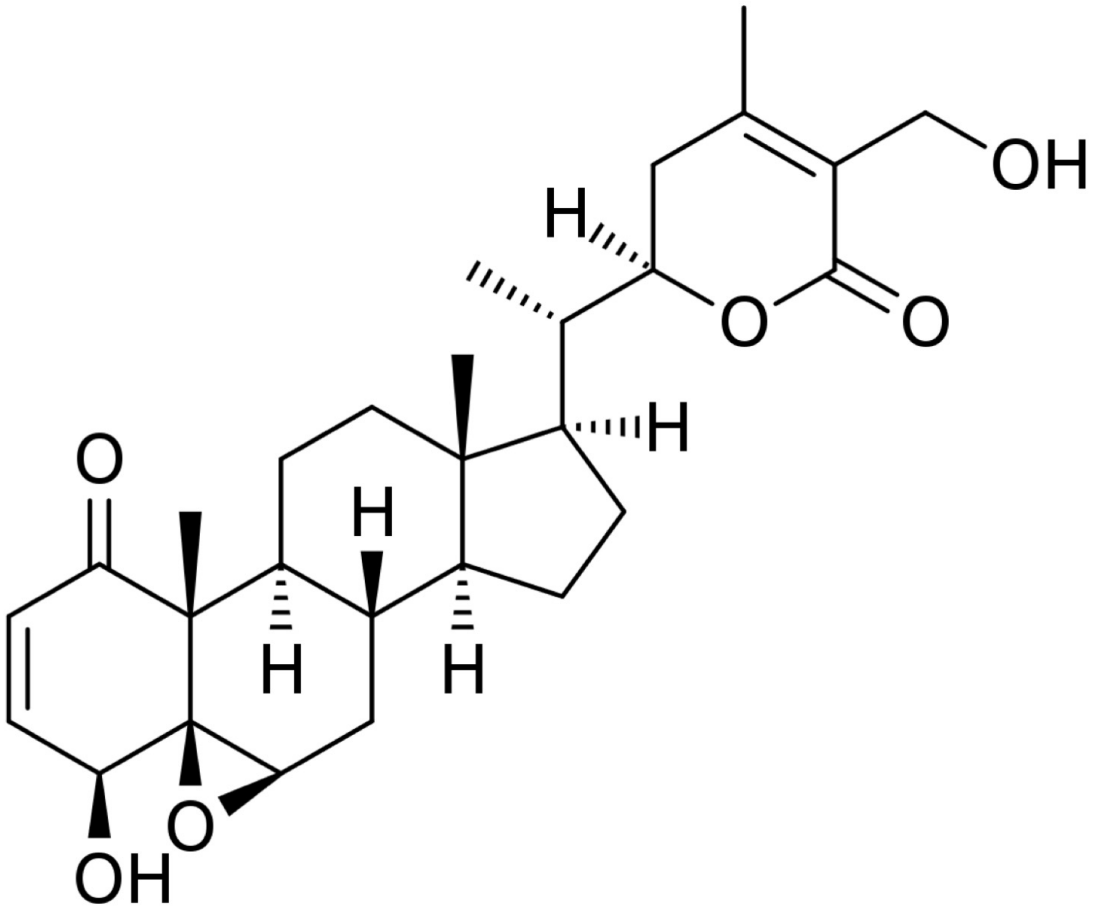

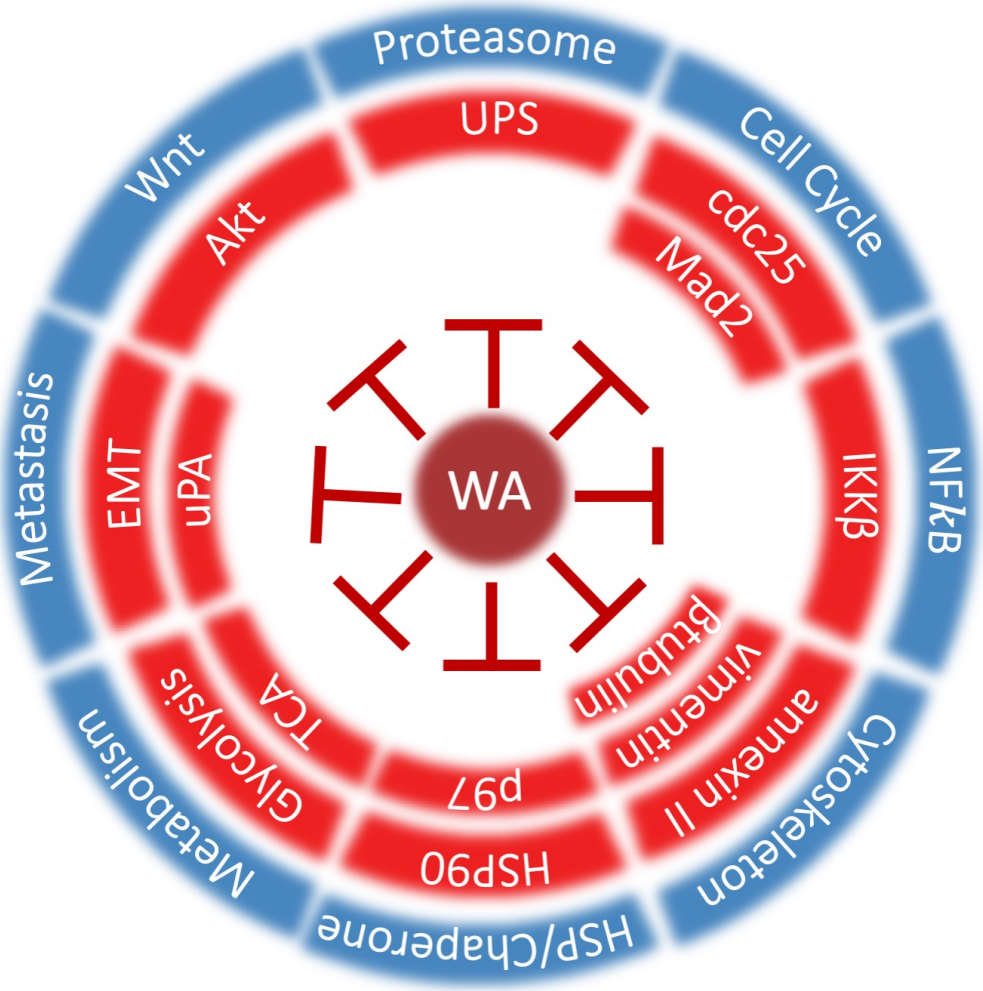
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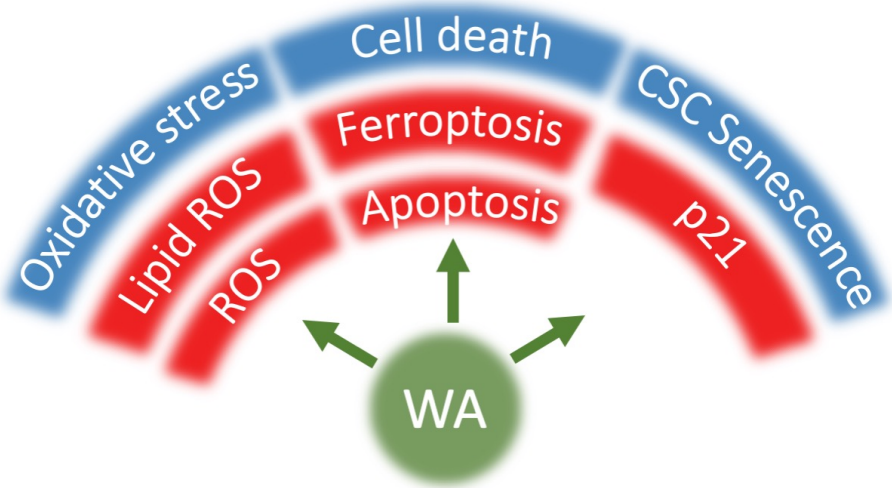
HSP90
Metabolism
HSP/Chaperone


Cell death
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