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REVIEW

Treatment of adult and pediatric high-grade gliomas with Withaferin A: antitumor mechanisms and future perspectives

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Abstract Resistance mechanisms employed by high-grade gliomas allow them to successfully evade current standard treatment of chemotherapy and radiation treatment. Withaferin A (WA), utilized in Ayurvedic medicine for centuries, is attracting attention for its antitumor capabilities. Here we review pertinent literature on WA as a high-grade glioma treatment, and discuss the cancerous mechanisms it affects. WA is relatively nontoxic and has shown potential in crossing the blood–brain barrier. WA prevents p53 alterations and inactivates overexpressed MDM2 through ARF and ROS production. Furthermore, WA upregulates *Bax*, inducing mitochondrial death cascades, inhibits mutated Akt, mTOR, and NF-κB pathways, and inhibits angiogenesis in tumors. Therapy with WA for high-grade gliomas is supported through the literature. Further investigation is warranted and encouraged to fully unearth its abilities against malignant gliomas.

Keywords Brain cancer · High-grade glioma · Withaferin A · Experimental therapy · Chemotherapy · Mechanisms of action · Astrocytoma · Glioblastoma

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Introduction

Withania somnifera (*W. somnifera*), indigenously referred to as Ashwagandha, is a medicinal plant commonly utilized in Ayurvedic medicine known for its sedative, rejuvenative, and life-prolonging properties [1]. Extracts from various parts of *W. somnifera* have been linked to different biological properties, such as anti-inflammatory, anti-bacterial, and anti-cancerous effects. One of the more widely studied constituents of *W. somnifera* is Withaferin A (WA) [2–4]. Derived from the leaves and roots of *W. somnifera*, WA is a steroidal lactone, containing four cycloalkane rings, in which the lactone ring consists of five carbon atoms and a single oxygen atom. Biochemical studies revealed that the anti-cancer functionality of WA is due mainly to multiple reactive sites within the compound, including hydroxyl and ketone moieties [4, 5]. This compound has shown anti-cancer efficacy in vitro by targeting cellular pathways involved in cell proliferation, growth, survival, and angiogenesis [5]. WA therefore contains promise as a non-toxic and effective adjuvant therapy for cancer patients. We sought to investigate and review the potential efficacy of WA on brain cancer, particularly adult and pediatric high-grade gliomas (HGGs).

HGGs account for around 70 % of all malignant primary brain cancers diagnosed in American adults, with an annual incidence of approximately five new cases per 100,000 people [6, 7]. Presently, the standard of care of newly diagnosed gliomas includes maximal safe resection with concurrent administration of the alkylating cytotoxic agent temozolomide (TMZ) and fractioned 60 Gy radiation therapy (RT) [8]. Despite this treatment, nearly all malignant gliomas recur. Although disease progression or recurrence has multiple specific treatment options, these tumors are associated with high morbidity and mortality

rates: median survival is around 15 months in patients with glioblastoma (GBM), and 2–5 years with anaplastic astrocytoma (AA) [9, 10]. In contrast to adult HGGs, pediatric HGGs, including anaplastic astrocytoma, glioblastoma, and diffuse intrinsic pontine gliomas, comprise approximately 8–12 % of all central nervous system (CNS) tumors diagnosed in children. These tumors are also highly aggressive, difficult to treat, and result in high morbidity and mortality rates. Furthermore, current treatment modalities for these pediatric brain tumors, including RT and chemotherapy (CT), demonstrate long-term effects on development, such as cognitive dysfunction, neuroendocrine dysregulation, and developmental delays [11, 12]. The dismal medial survival time has been attributed to the existence of the multiple drug resistance capabilities of HGGs (e.g., treatment resistant cancer stem cells, up-regulation of drug resistant genes, pro-survival responses such as autophagy, etc.) [13]. Treatment focus now relies heavily on agents with low toxicity profiles to effectively penetrate the CNS [14]. There is critical need for the emergence of innovative chemotherapeutic agents in the management of adult and pediatric HGGs that can act singularly or in synergistic combinations with other treatment modalities—a need that WA can potentially fill.

Here, we provide a brief overview of treatment resistance mechanisms adopted by HGGs, and common pathways that are altered in HGGs. Additionally, we offer a comprehensive review of WA as a potential adjunct therapy by discussing cellular responses induced within tumor tissues. By including the mechanisms of action of WA in pathways often deregulated in brain cancer, we look to provide a future perspective of WA treatment against gliomas.

Characteristics of HGGs

Mechanisms of resistance to CT and RT

The failure of the current chemoradiotherapy to eliminate all glioma cells is due to the presence of multiple resistance mechanisms. It is speculated that developed resistance to current therapy leads to 90 % of HGGs recurring at the primary site [8, 9, 15].

One such resistance mechanism is the presence of glioma stem-like cells (GSLCs)—a subset of tumor cells implicated in disease recurrence. GSLCs confer a therapeutic challenge because of their self-renewal properties and preferential activation of DNA repair machinery due to insults from CT and RT [13, 16]. RT kills tumor cells primarily through DNA damage; thus, DNA damage checkpoints play a crucial role in cellular radiosensitivity. GSLCs phosphorylate the DNA repair machinery, such as the ataxia-telangiectasia-mutated (ATM), Rad17, Chk1 and Chk2 checkpoint proteins, to a higher extent than non-stem-like cells, indicating that GSLCs have the ability to induce higher rates of DNA repair post-RT [13].

Other resistance mechanisms include the up-regulation of multi-drug resistant genes (e.g., BCRP1), DNA-repair enzymes (e.g., *O*-6-methylguanine-DNA methyltransferase, MGMT), and antiapoptotic factors (e.g., Survivin), that increase difficulty in effective tumor management [17, 18]. MGMT works by counteracting the ability of TMZ to introduce cytotoxic methyl adducts, such as *O*-6-methylguanine (*O*-6-meG), which promote DNA strand breakage and subsequent cell death by interfering with successive cycles of DNA replication [19]. MGMT restores the structural integrity of bases with *O*-6-meG by transferring the added methyl group to a cysteine residue within its own active site. Naturally, its expression has been show to confer chemoresistance against TMZ in vitro and in vivo [18, 19]. Expression of Survivin, a member of the inhibitor of apoptosis (IAP) family, has been shown to correlate with treatment resistance in HGGs [20]. The importance of Survivin is illustrated in studies where Survivin disruption abolishes GSLC survival and growth [21]. Furthermore, pro-survival responses, such as autophagy, promote cell survival by allowing tumor cells to neutralize the effects of cytotoxic therapies. Lin et al. [22] showed TMZ-induced cytoprotective autophagy through a reactive oxygen species (ROS) burst and extracellular signal-regulated kinase (ERK), and, once autophagy was inhibited, malignant glioma cells underwent apoptosis. Knizhnik and colleagues [23] verified TMZ-induced autophagy is a survival mechanism, stimulating senescence rather than apoptosis.

Resistance mechanisms and deregulated pathways commonly seen in HGGs are summarized in Fig. 1.

Origin and characteristics of Withaferin A

Structure/anticancer properties and active sites

As previously mentioned, WA is derived from the roots and leaves of the *W. somnifera* plant—an erect, evergreen shrub distributed throughout India. Withanolides are a group of naturally occurring C28-steroidal lactones isolated from the roots and leaves of *W. somnifera*. Among them, WA is one of the most bioactive compounds, possessing anti-inflammatory, pro-apoptotic, and anti-invasive, as well as anti-angiogenic, effects. However, the chemical mechanisms by which WA accomplishes these activities are still largely unknown. Acylation or alkylation of critical macromolecules and enzymatic active sites by covalent bonds are among the several proposed explanations [24]. Chemical structural analysis of WA suggests

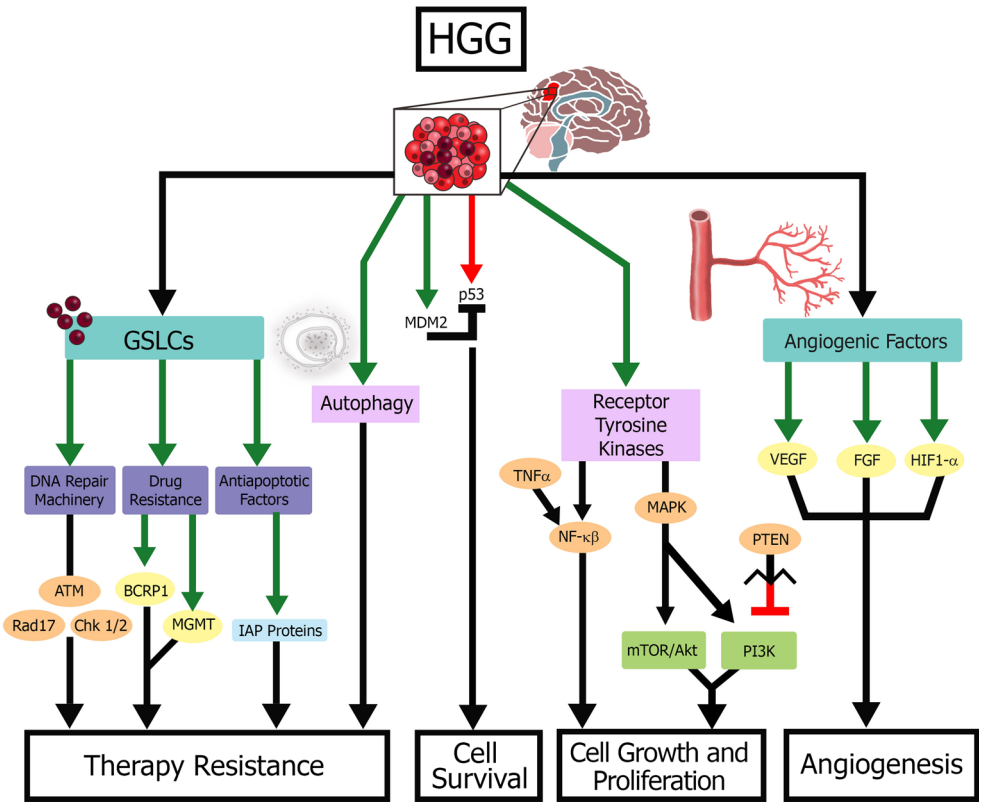

Fig. 1 Diagram of the various pathways deregulated in high-grade gliomas (HGG) cells, leading to overall tumor cell survival. *Green arrows* Upregulation of signaling pathway, *red lines* decreased expression or downregulation of signaling pathway (color figure online)

three positions that might be involved in the alkylation reactions with nucleophilic sites. These include the C3 of the α,β-unsaturated ketone in ring A; the epoxide at position C5,6; and the C24 of the α,β-unsaturated lactone in ring E. Carbon 3 in the unsaturated A-ring has been identified by NMR spectral analysis as the main nucleophilic target site for ethyl mercaptan, thiophenol and L-cysteine ethyl ester in vitro [6].

Structure–activity relationship (SAR) studies have indicated that removal of the double bond in ring A considerably decreased the activity, indicating that the presence of the double bond in WA contributes significantly to its activity. In another example, disruption of ring A prevented the ability of WA to bind to and induce vimentin fragmentation, reversing WA's inhibition of cancer cell invasion and apoptosis [25]. Introduction of a bulky group, such as glucose, at the C27 hydroxyl group caused a reduction in activity. This suggested that the C27 hydroxyl is important for activity. However, introduction of small groups, such as acetates, did not affect activity [2]. Inactivation of the 5(6) epoxide group with 2-mercaptoethanol resulted in the loss of activity, indicating the requirement of this group for biological activity [26]. For example, SAR studies determined that this group was important for binding and inhibiting the molecular chaperone, HSP90, resulting in HSP90 client protein degradation and pancreatic cell death [27]. Furthermore, the authors of the latter study suggested that the hydroxyl group at C4 may increase binding to HSP90 and disrupt its interaction with the key co-chaperone Cdc37. Therefore, based on SAR studies and on the chemical reactions performed, it is reasonable to conclude that biological activities of these withanolides was due mainly to an α,β-unsaturated ketone moiety in ring A, C5(6) epoxide, and hydroxyl group at C27 (Fig. 2).

Blood–brain barrier

An important limitation to current treatment for HGGs is the inability of antitumor chemotherapeutics to penetrate the blood–brain-barrier (BBB). Therefore, in order to be considered as a possible HGG treatment, WA must also be able to cross the BBB effectively. One preliminary study showed that WA treatment led to a survival advantage in a murine orthotopic GBM model, suggesting that the WA compound, or one of its active metabolites, is able to reach the brain tumor [28, 29].

Additionally, WA does not violated the Rule of Five, otherwise known as Lipinski's Rule of Five, which assesses a compound's properties to determine the likelihood of its efficacy as an orally absorbed drug. Lipinksi and colleagues analyzed the physicochemical properties of roughly 2000 drugs, and concluded compounds were more likely to be membrane permeable if they adhered to a set of criteria [30]. These criteria have also been applied to

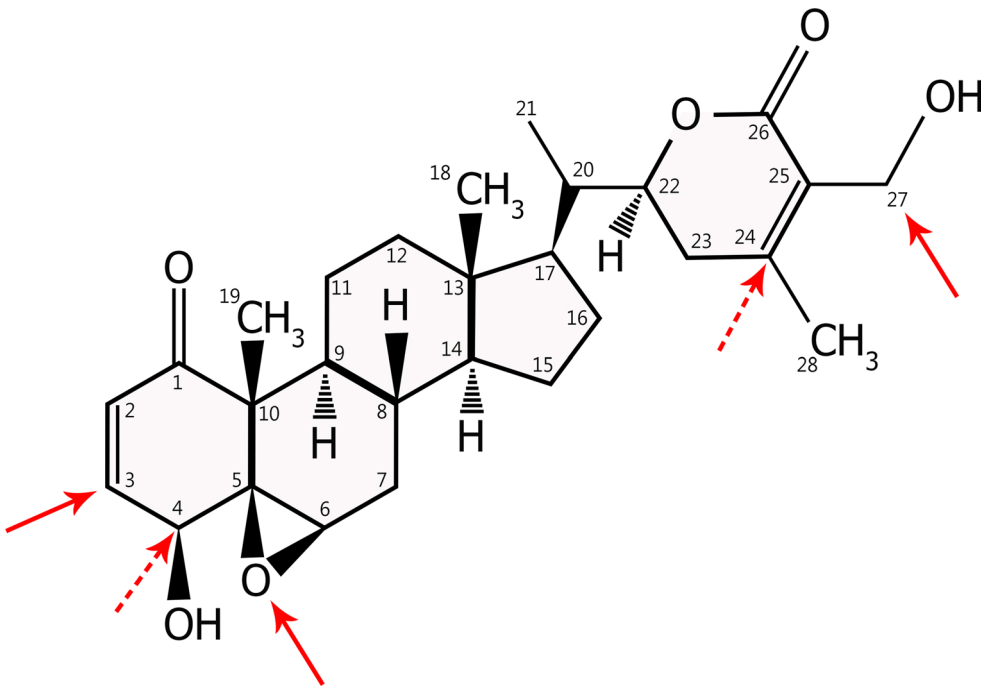

Fig. 2 Chemical structure of Withaferin A (WA) with *arrows* indicating pertinent reactive sites with anticancer properties. *Solid arrows* Essential sites, *dashed arrows* sites in which modification may affect biological activity. Molecular formula, $C_{28}H_{38}O_6$; molecular mass, 470.6 g/mol

Table 1 Withaferin A (WA) does not violate any condition set forth by Lipinski's rule of five [30], which assesses the molecular likelihood of membrane permeability

Lipinski's rule of five[a]	Properties of WA	Adherence to rules
(1) Molecular weight under 500 Da	Molecular weight = 470.6 Da	Yes
(2) 1-Octanol/water partition coefficient[b] (log *P*) not greater than 5	Partition coefficient = 3.50 [33]	Yes
(3) No more than five hydrogen bond donors (total number of nitrogen–hydrogen and oxygen–hydrogen bonds)	H-bond donors = 2	Yes
(4) Not more than ten hydrogen bond acceptors (all nitrogen or oxygen atoms)	H-bond acceptors = 6	Yes

[a] Lipinski's rule of five refers to the four criteria of membrane permeability whose numeric constraints have a common denominator of five

[b] The compound's lipophilicity, expressed as a quantity known as log *P* (the logarithm of the partition coefficient between water and 1-octanol)

characterize the ability of drugs to pass through the BBB [28, 31]. WA does not display any violation of these rules, and contains a favorable blood/brain partition coefficient, which implies its efficacy in penetrating the BBB (Table 1) [32]. Additional in vivo studies are needed in mammals to determine WA's effectiveness in crossing the BBB to truly ensure its potential for success in human trials.

Safety considerations

The anticancerous benefits of chemotherapeutic agents must be balanced against their inherent cytotoxic properties to normal cellular tissues. Ideally, a chemotherapeutic agent targets cancer cells without affecting the integrity of surrounding tissues. WA has been shown to selectively induce cell death in cancerous cells while sparing non-cancerous cells [25, 34, 35]. A study was conducted to elucidate the effects of WA on prostate adenocarcinoma cells as compared to WA effects on normal human fibroblasts. After a 2 μM treatment of WA, normal cells were found to be still viable in vitro at 96 h, whereas cancerous cells demonstrated a loss of viability at 72 h [34]. In vivo experiments also show that WA has little to no cytotoxic effects on normal cells. Thaiparambil et al. [25] discovered that, when WA was utilized to treat breast cancer in mice, histological sections of lung and liver parenchyma from WA-treated mice versus non-treated mice showed minimal necrosis at doses up to 4 mg/kg. Such experiments imply that WA spares normal tissue from the negative effects seen in cancer cells.

It important to note the dose-dependent effects of WA in human cancers remain to be determined. Further investigation is warranted to fully elucidate the effects of WA in humans; however, the compound has been shown to be safe in preliminary in vitro and in vivo studies.

Anti-HGG properties of WA

WA as an effective treatment against known resistance mechanisms

GSLCs play a huge role in tumor renewal due to their self-protective properties when targeted by CT and RT, so the importance of effectively treating this subpopulation of tumor cells cannot be understated. Studies with mice bearing human ovarian tumors have shown that WA alone can preferentially target putative cancer stem-like cells (CSLCs), causing dose-related cell death [36, 37]. Furthermore, combining WA with cisplatin resulted in a synergistic effect, reducing tumor size by 70–80 % and preventing metastasis [36]. The latter is displayed in WA's ability to inhibit epithelial-mesenchymal transition by suppressing vimentin, a key protein in mesenchymal mobility [38]. Indicators of CT resistant GSLCs, such as aldehyde dehydrogenase 1 (ALDH1) activity, and stem cell marker gene expression (CD44, CD24 and CD 117) were significantly decreased when exposed to WA [37]. Notch1 signaling, which plays an important role in cancer stem cell self-renewal, was also inhibited upon WA treatment, resulting in inhibition of Notch1 downstream signaling genes Hes1 and Hey1. These genes normally function to prevent differentiation of quiescent cells, aiding cancer progression. Whether in monotherapy or in combination effect with cisplatin, WA treatment also resulted in an increase in ROS production and DNA damage, therefore causing cell death and apoptosis in GSLCs [37]. These results reveal the ability of WA to overcome and sensitize the protective barriers exhibited by GSLCs.

Furthermore, TMZ-resistant HGGs express MGMT, which inhibits *O*-6-meG adducts from inducing DNA strand breakage, thereby promoting cell survival. Current research looks at combining WA and TMZ, which shows promise by allowing resistant cells to become sensitized and undergo apoptosis [39, 40]. WA treatment acts by depleting MGMT, enabling TMZ to cause DNA damage, G2/M cell cycle arrest, and death in cancerous cells [41]. The TMZ resistant cell line T98G underwent a 43 % MGMT reduction after treatment with 5 μM WA, and completely eliminated MGMT production at 10 μM WA. This study demonstrates the inverse correlation between MGMT production and TMZ effectiveness, and also provides evidence of the ability of WA to sensitize HGG cells to treatment [40].

Pro-survival proteins, such as Survivin, are also inhibited by WA, which reduces cancer cell viability. The effect of WA on Survivin was tested in in vivo studies using hamsters with induced oral carcinogenesis as well as in human breast cancer xenografted mice [41]. After 6 h of up to 5 μM of WA exposure, human breast cancer cells displayed a decrease in Survivin, XIAP, and cIAP-2 protein levels.

WA targets deregulated pathways common in HGGs

p53 signaling pathway

The p53 pathway is one of the pathways most commonly mutated in tumorigenesis with a low survival rate [42]. It acts as a tumor suppressor, modulating cell cycle arrest and apoptosis in response to cytotoxic stress and DNA damage. In addition to its tumor-suppressing functions, it acts as a transcription factor that regulates genes involved in tumor development, proliferation, and infiltration [43]. Mutations in p53 can be found in up to 80 % of adult HGG patients and approximately 50 % of pediatric HGGs [44, 45]. Mutations to p53 affect its ability to induce pro-apoptotic pathways when genetic anomalies are detected, thereby propagating the genomic instability of glioma cells [44, 46].

Panjamurthy et al. [47] found that oral administration of WA (20 mg/kg for 14 weeks) entirely prevented 7,12-dimethylbenz(a)anthracene(DMBA)-induced oral squamous cell carcinoma in golden Syrian hamsters. Furthermore, in an immunohistochemical analysis, DMBA-painted animals stained 80 % positive for p53 mutation, while WA-treated DMBA-painted animals showed a significantly weaker positive staining for p53 mutation. While this study was performed on hamsters with oral cancers, the results highlight that WA may preserve the integrity of p53 proteins during tumorigenesis.

Furthermore, an indirect mutation of p53 results in the amplification of MDM2—an E3 ubiquitin ligase that disrupts p53 activity by transcription inhibition, binds to p53 active sites, and marks it for degradation. Around 15 % of AA and GBMs display amplification of MDM2, indicating a means for tumors to deactivate p53 other than by direct mutation [26]. Increased concentrations of MDM2 within cellular structures can exert tumorigenic phenotypes. Currently, new anticancer strategies focus on inactivating MDM2 to restore p53 function. Some therapies, such as Cisplatin administration, are dependent on regular p53 function [48]. WA is able to stabilize p53 through activating ARF, which functions to prevent MDM2 binding to p53 [49].

Additionally, cancer cells with mutated p53 proteins are often ROS-compromised; the ubiquitin proteasome system (UPS) is deregulated in the removal of oxidized proteins, allowing pro cell survival [49, 50]. In addition to targeting the UPS, investigators also studied the activation of TAp73, a tumor suppressor that causes chemosensitivity to Cisplatin or CT when p53 is lost or mutated [49]. TAp73 is also modulated through MDM2 concentrations in a relationship similar to the p53/MDM2 complex. WA-induced ROS activates JNK kinases to phosphorylate TAp73, thereby inhibiting the TAp73/MDM2 complex. The synergistic effect of WA-induced ROS production and the inhibition of the TAp73/MDM2 complex allowed TAp73 to induce apoptosis in cancer cells [49].

MAPK/ERK pathway

The MAPK/ERK pathway is a chain of proteins that participate in communication of signal molecules on cell surface receptors to nuclear DNA to promote cellular growth and proliferation or cell death; this pathway is frequently mutated in human cancer. Extracellular mitogens attach to a cell-surface receptor, causing the activation of Rat sarcoma protein (Ras). From here, activated Ras effects downstream activation of Raf, MEK, and, finally, ERK, to elicit cellular responses such as increased growth and cell mitosis [51, 52]. Deregulation of any point in the pathway can induce an increase in cellular proliferation, signal transduction, apoptosis, and tumorigenesis. HGGs up-regulate the MAPK/ERK pathway by producing a greater number of cell signal receptors, such as epidermal growth factor receptors (EGFR), which can be seen in 40–60 % of adult cases [53] Increased levels or increased activity of these receptors leads to amplified signal transduction and an enhancement in tumorigenesis.

WA has been shown to have many functions in the cascade, usually with p38MAPK, which functions to induce RNA interference against apoptosis [32, 35, 54]. In one case with in vitro leukemic cells from clinical patients before CT, WA phosphorylated p38MAPK, which phosphorylated ATF-2 and HSP27, ultimately leading to an increase in Bax, promoting the mitochondrial death cascade [35]. The results showed an increase in phosphorylation of p38MAPK of ~90.96 %with a 3.0 μM treatment of WA after 2.5 h. After 72 h of WA treatment, no viable leukemic cells of myeloid origin were observed.

PI3K/Akt/mTOR pathway

PI3K phosphorylation activates Akt, which then activates many downstream targets, mainly mTOR [55]. Additionally, up-regulation in Akt or its hyperactivation (due to overexpression of growth factor receptors like EGFR) has been seen to promote uncontrolled cell cycle progression in HGG cells and their subsequent protection from apoptosis. Numerous studies have found that WA produces an inhibitory effect on Akt and mTOR signaling [32, 56, 57]. Multiple oncogenic pathways, including MAPK, Akt, NF-κB, and mTOR often associate directly with Notch signaling. Overexpression of Notch-1 resulted in an increase of expression in Akt/mTOR signaling in colon cancer lines, promoting cell survival [56]. Reduction of Notch-1 reduced Akt/mTOR signaling. Western blot analysis revealed that treatment with 5 μM WA treatment reduced phosphorylated Akt (pAkt) as well as Notch-1 expression significantly over a period of 24 h. However, total Akt levels remained the same throughout the entire treatment. The WA inhibition of Notch-1 helped facilitate significant JNK-mediated apoptosis in colon cancer cells. After WA treatment, the cell percent viability of three colon cancer cell lines SW-480 (IC_{50}: 3.56 μM), SW-620 (IC_{50}: 5.0 μM) and HCT-116 (IC_{50}: 5.33 μM) also showed a significant decrease.

Another study revealed WA (2 μM) in combination with oxaliplatin (25 μM) reduced both pAkt and total Akt levels over 24 and 48 h [58]. In non-cancerous cells, the activity of PI3K is counteracted by its inhibitor, and tumor suppressor, phosphatase and tensin homolog (PTEN) [60]. However, in more than 60 % of GBMs and other HGGs, PTEN is inactivated by genetic alterations, allowing PI3K to act uninhibited [59]. Phosphorylated PTEN levels were also reduced in combination therapy with no significant change in total PTEN levels [58].

NF-κB pathway

The NF-κB family of transcription factors plays an important role in inflammatory and immune responses. It is found in almost all animal cell types and is involved in cellular responses to various stimuli such as stress, radiation, and free radicals [61, 62]. A common regulatory step in this signal cascade is the activation of IκB kinase (IKK), which prevents NF-κB function. Activation of NF-κB dimers is due to phosphorylation of IκB by IKK, enabling the active NF-κB transcription factor subunits to translocate to the nucleus and induce target gene expression [63, 64]. Deregulation of the NF-κB pathway is involved in HGGs through the up-regulation of factors that activate NF-κB, such as tumor necrosis factor-α (TNFα) [65, 66].

An in vitro and in vivo study in mice revealed that WA prevented TNF from activating downstream signaling of IκB kinase β via a thioalkylation-sensitive redox mechanism [67]. WA inhibited TNF-induced NF-κB activation in murine fibrosarcoma and human embryonic kidney cell lines [37, 67]. Another study showed that WA increased

ubiquitinated-proteins in TNF-α-treated human umbilical vein endothelial cells at global levels, which suggests that WA tends to target the ubiquitin–proteasome pathway (UPP) [50, 68].

Effects on angiogenesis

HGGs are among the most angiogenic of cancers: one of the hallmarks of transformation from low-grade glioma (LGG) to HGG is the stimulation of angiogenesis and the formation of new vessels [67]. Angiogenesis supplies the cancerous microenvironment with new vasculature from pre-existing blood vessels. This process is coordinated by an increase in pro-angiogenic gene expression, including vascular endothelial growth factor (VEGF) [68]. Due to the importance of angiogenesis in HGG progression, greater attention has been paid to anti-VEGF therapies. While enthusiasm for evaluating anti-VEGF agents has been relatively dampened by safety concerns, including the risk of intracranial hemorrhage, recent trials among malignant glioma patients treated with VEGF- or VEGF receptor (VEGFR)-targeting therapeutics plus CT report an antitumor benefit, as well as acceptable safety profiles [69].

WA shows potential for inhibiting angiogenesis by binding VEGF. One study analyzed WA's potential for molecular docking through programs such as SwissDock and Docking Server [70]. Current anti-angiogenic therapies approved by the FDA, such as Bevacizumab, have serious to life-threatening side effects, and have little effect on brain tumor invasiveness [71–73]. After treatment with Bevacizumab, glioblastomas will adapt by upregulating glycolysis production, moving towards a more anaerobic metabolism, and inducing a microenvironmental acidosis. This adaptation leads to tumor growth, invasiveness, and unconstrained proliferation. In comparison to Bevacizumab, WA showed higher cluster formation and an overall more favorable binding and affinity to VEGF using Fullfitness [70]. An in-depth study that combined Withanone, a compound found in *W. somnifera* (1 mg/kg) and Withaferin A (0.5 mg/kg) (WiNA), tested both in vitro and in vivo [74]. The in vivo studies consisted of human glioblastoma, osteosarcoma, fibrosarcoma, neuroblastoma, rat glioblastoma, and mouse fibroblast cell lines. WiNA was toxic to cancer cells and nontoxic on normal human cells at the optimal ratio of 20:1. Ratios such as 5:1 and 3:1 became toxic to normal cells as well. In VEGF-stimulated HUVECs, WiNA limited migration and invasiveness. In vivo studies with nude mice subcutaneous xenograft and lung metastasis models revealed nontoxicity in mice as well as tumor and VEGF

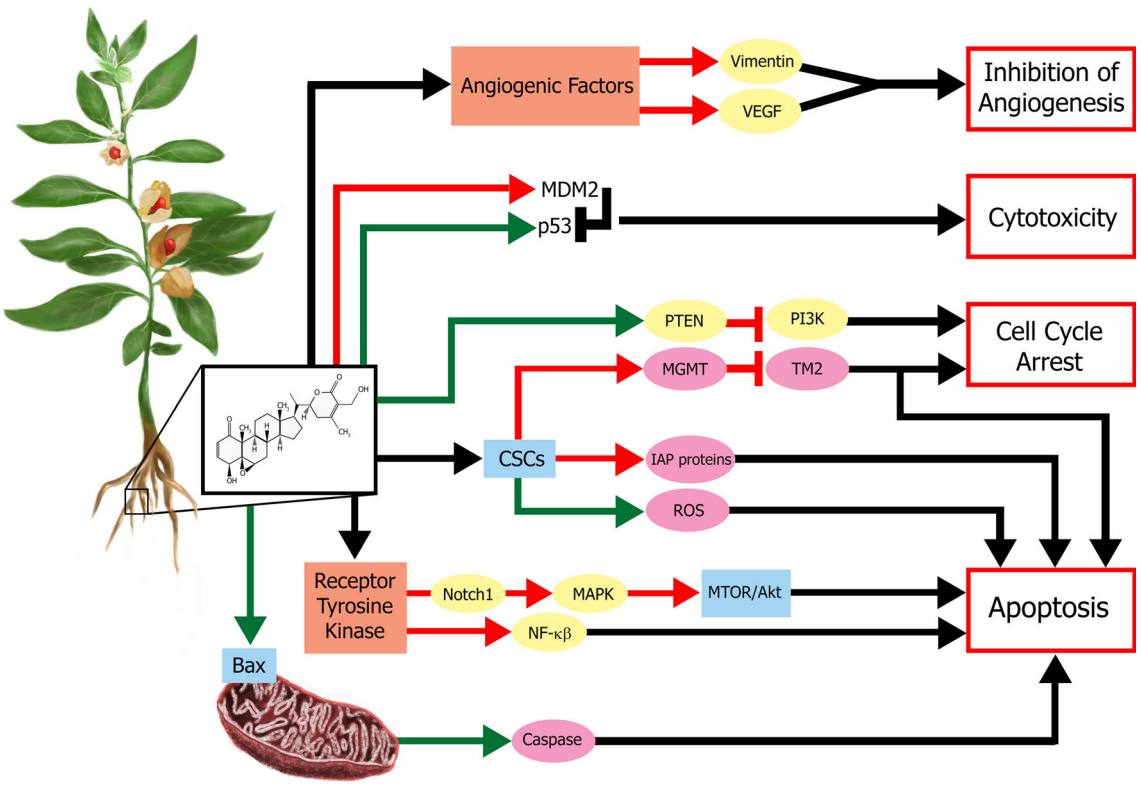

Fig. 3 A diagram of the effects of WA on various pathways in HGG cells, leading to tumor cell death and inhibition. *Green arrows* Increased protein expression, *red arrows* decreased protein expression, *red bars* inhibition of downstream signaling targets (e.g., PTEN inhibits PI3K) (color figure online)

Table 2 Studies in the literature that include WA as a treatment against high-grade gliomas (HGGs)

References	Study type	Biological context	HGG type	Pertinent conclusions
Grogan et al. [78]	Laboratory research	In vitro	GBM	In GBM, WA inhibits proliferation through G2/M cycle arrest, inhibits Akt/mTOR pathways, increases ROS production, upregulates HSP, and downregulates HSF1
Grogan et al. [39]	Laboratory research	In vitro	GBM	Along with previous results (mentioned above), WA with TMZ causes dose dependent depletion of MGMT, which sensitizing GBM cancer cells to cytotoxic effects of TMZ
Shah et al. [79]	Laboratory research	In vitro	Glioma	WA inhibited proliferation, enhanced expression of GFAP, induced senescence-like growth arrest, and delayed cellular migration

GFAP Glial fibrillary acidic protein, *GBM* glioblastoma multiforme, *HSF1* heat shock factor, *HSP* heat shock proteins, *ROS* reactive oxygen species, *TMZ* temozolomide

reduction in small tumors. This combination effect using multiple parts of *W. somnifera* may be another therapy worthy of future study in HGGs.

At low doses, WA exerts potent anti-angiogenic activity in vivo [66, 75]. Treating human umbilical vein endothelial cells (HUVEC) with WA resulted in induced cytostatic cell cycle G_1 arrest. WA also inhibited vessel formation in vitro in endothelial cells and in vivo in mice-induced tumors [66, 76]. WA also has the ability to bind to vimentin and covalently modify its cysteine residue, which is present in the highly conserved α-helical coiled-coil 2B domain. This binding causes an aggregation of vimentin, ultimately leading to apoptosis [76]. WA suppressed neovascularization in wild type mice at 73 % inhibition and only marginally inhibited (29 %) neovascularization in vimentin-null mice. This revealed that inhibition of capillary growth from WA treatment relies on vimentin expression.

A summary of cellular responses induced by WA can be found in Fig. 3.

Future perspectives

Recent laboratory investigations have begun to elucidate the multitude of effects WA exerts on cancer cells while simultaneously having minimal consequences on normal tissue. WA in various cancer types primes it to be efficacious in HGGs because many deregulated signaling pathways commonly identified in HGGs are also characteristically preserved in other cancer types [77]. Unfortunately, there is little information available on WA as a treatment against HGGs. In the literature, there are a few papers investigating the use of WA directly against HGGs (Table 2). Along with these other authors, we believe that WA can be applied to treating HGGs, and that WA therapy has the potential to yield remarkable results.

Interestingly, WA has been found to have promising effects in multiple other disease entities. In many cases, safety has already been shown and studies are now investigating efficacy. Table 3 summarizes these trials and each of their focuses.

The future of utilizing WA for HGG treatment involves determining its ability to cross the BBB in humans. WA adheres to Lipinski's Rule of Five; therefore, pharmacokinetic study could confirm the ability of WA to be BBB permeable. Additionally, more in vivo studies are needed to allow scientists to gain additional evidence of WA safety in relevant biological contexts. Overall, the future perspectives behind the use of WA in HGGs are positive. The innocuous nature and efficacy of WA in completed clinical and preclinical trials make it a compound highly worthy of consideration for the treatment of pediatric HGGs. A great deal of preliminary work has emerged to solidify WA's place as an efficient anti-cancer treatment. Its success across various tumor types justifies its use in brain cancer settings.

Conclusions

Targeting HGGs requires a novel approach in response to tumor resistance to current treatment modalities, and the multitude of deregulated signaling pathways characteristic of malignant brain cancers. Metabolites from the indigenous Indian plant *W. somnifera* have garnered attention from the scientific community for their novel cancer preventative and anti-tumor properties. Specifically, WA derived from the root of *W. somnifera* has shown its anti-cancer efficacy in humans across multiple cancer types, and its effects should warrant its further study in HGGs. WA is hypothesized to be BBB permeable, allowing for infiltration into the brain, which further strengthens its potential for use in the setting of HGGs.

WA effectively targets and prevents over-proliferation of GSLCs, while downregulating survival proteins expressed in HGGs, such as Survivin. In preclinical studies, WA was able to prevent alterations to p53 in a dose-dependent manner, as well as inactivate MDM2 through activation of ARF and ROS production. Additionally, WA promotes a mitochondrial death cascade by upregulating

Table 3 Clinical trials that include WA as a treatment/drug for various conditions

Sponsor	Status	Type	Estimated enrollment	Clinicaltrials.gov identifier	Study start date	Estimated study completion date	Condition treated
University of Pittsburgh	Completed	Phase III	60	NCT00761761	October 2008	March 2011	Bipolar I/II disorder; bipolar disorder NOS
University of Pittsburgh	Recruiting	NA	80	NCT01793935	April 2013	March 2016	Schizophrenia; schizoaffective disorder
Natreon, Inc.	Completed	Phase II	120	NCT01311180	March 2011	November 2012	Generalized anxiety disorder
Tata Memorial Hospital	Unknown	Phase I/II	24	NCT00689195	May 2008	June 2013	Osteosarcoma
National College of Natural Medicine	Completed	Phase I	25	NCT00817752	May 2007	February 2008	Inflammation; cancer; autoimmune diseases
Vedic Lifesciences Pvt. Ltd.	Completed	NA	23	NCT02027467	February 2014	April 2014	Preventive health measures
National Center for Complementary and Integrative Health (NCCIH)	Completed	Phase II	NA	NCT00010634	September 1999	July 2004	Periodontitis
SomaLogic, Inc.	Withdrawn	NA	28	NCT01125501	April 2010	June 2010	Metabolic syndrome
University of Colorado, Denver	Completed	NA	70	NCT00977730	July 2008	July 2011	Non-alcoholic steatohepatitis
Tel-Aviv Sourasky Medical Center	Completed with results	Phase IV	30	NCT00719953	August 2008	August 2009	Elderly memory impairment
University of Louisville	Active	NA	40	NCT02172625	November 2014	July 2015	Oxidative damage

Bax, inhibits mutated Akt, mTOR, and NF-κB pathways, and inhibits angiogenesis in tumors. However, while its effects are promising in preclinical settings, further investigation is warranted to fully unearth the ability of WA to act against HGGs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no financial or other conflicts of interest in relation to this research and its publication.

References

1. Mirjalili MH, Moyano E, Bonfill M et al (2009) Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. Molecules 14(7):2373–2393
2. Jayaprakasam B, Nair MG (2003) Cyclooxygenase-2 enzyme inhibitory withanolides from *Withania somnifera* leaves. Tetrahedron 59(6):841–849
3. Owais M, Sharad KS, Shehbaz A, Saleemuddin M (2005) Antibacterial efficacy of *Withania somnifera* (ashwagandha) an indigenous medicinal plant against experimental murine salmonellosis. Phytomedicine 12(3):229–235
4. Sangwan NS, Sabir F, Mishra S et al (2014) Withanolides from *Withania somnifera* Dunal: development of cellular technology and their production. Recent Pat Biotechnol 8(1):25–35
5. Joshi P, Misra L, Siddique AA, Srivastava M, Kumar S, Darokar MP (2014) Epoxide group relationship with cytotoxicity in withanolide derivatives from *Withania somnifera*. Steroids 79:19–27
6. Vanden Berghe W, Sabbe L, Kaileh M et al (2012) Molecular insight in the multifunctional activities of Withaferin A. Biochem Pharmacol 84(10):1282–1291
7. Stupp R, Brada M, van den Bent MJ et al (2014) High-grade glioma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 25(Suppl 3):iii93–iii101
8. CBTRUS 2008 Statistical report: primary brain tumors in the United States, 1998–2002. Central Brain Tumor Registry of the United States, 2000–2004. http://www.cbtrus.org/reports/2007-2008/2007report.pdf. Accessed 7 July 2008
9. Bleeker FE, Molenaar RJ, Leenstra S (2012) Recent advances in the molecular understanding of glioblastoma. J Neurooncol 108(1):11–27
10. Wen PY, Kesari S (2008) Malignant gliomas in adults. N Engl J Med 359(5):492–507
11. Armstrong GT, Liu Q, Yasui Y et al (2009) Long-term outcomes among adult survivors of childhood central nervous system malignancies in the Childhood Cancer Survivor Study. J Natl Cancer Inst 101(13):946–958

12. Shah SS, Dellarole A, Peterson EC, Bregy A, Komotar R, Harvey PD, Elhammady MS (2015) Long-term psychiatric outcomes in pediatric brain tumor survivors. Child Nerv Syst 31(5):653–663
13. Omuro A, DeAngelis LM (2013) Glioblastoma and other malignant gliomas: a clinical review. JAMA 310(17):1842–1850
14. Nageswara Rao AA, Scafidi J, Wells EM, Packer RJ (2012) Biologically targeted therapeutics in pediatric brain tumors. Pediatr Neurol 46(4):203–211
15. Bao S, Wu Q, McLendon RE et al (2006) Glioma stem cell promotes radioresistance by preferential activation of the DNA damage response. Nature 444(7120):756–760
16. Stupp R, Mason WP, Bent MJ et al (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352:987–996
17. Lathia JD, Mack SC, Mulkearns-Hubert EE, Valentim CL, Rich JN (2015) Cancer stem cells in glioblastoma. Genes Dev 29(12):1203–1217
18. Cho DY, Lin SZ, Yang WK et al (2013) Targeting cancer stem cells for treatment of glioblastoma multiforme. Cell Transplant 22(4):731–739
19. Johannessen TC, Bjerkvig R (2012) Molecular mechanisms of temozolomide resistance in glioblastoma multiforme. Expert Rev Anticancer Ther 12(5):635–642
20. Dahan P, Martinez Gala J, Delmas C et al (2014) Ionizing radiations sustain glioblastoma cell dedifferentiation to a stem-like phenotype through survivin: possible involvement in radioresistance. Cell Death Dis 5:e1543
21. Johannessen TC, Bjerkvig R, Tysnes BB (2008) DNA repair and cancer stem-like cells-potential partners in glioma drug resistance. Cancer Treat Rev 34(6):558–567
22. Guvenc H, Pavlyukov MS, Joshi K et al (2013) Impairment of glioma stem cell survival and growth by a novel inhibitor for Survivin-Ran protein complex. Clin Cancer Res 19(3):631–642
23. Lin CJ, Lee CC, Shih YL et al (2012) Resveratrol enhances the therapeutic effect of temozolomide against malignant glioma in vitro and in vivo by inhibiting autophagy. Free Radic Biol Med 52(2):377–391
24. Moncrief JW, Heller KS (1967) Acylation: a proposed mechanism of action for various oncolytic agents based on model chemical systems. Cancer Res 27(8):1500–1502
25. Thaiparambil JT, Bender L, Ganesh T et al (2011) Withaferin A inhibits breast cancer invasion and metastasis at sub-cytotoxic doses by inducing vimentin disassembly and serine 56 phosphorylation. Int J Cancer 129(11):2744–2755
26. Misra L, Lal P, Chaurasia ND, Sangwan RS, Sinha S, Tuli R (2007) Selective reactivity of 2-mercaptoethanol with 5β,6β-epoxide in steroids from *Withania somnifera*. Steroids 73(3):245–251
27. Gu M, Yu Y, Gunaherath GB, Gunatilaka L, Li D, Sun D (2014) Structure-activity relationship (SAR) of withanolides to inhibit Hsp90 for Its activity in pancreatic cancer cells. Invest N Drugs 32(1):68–74. doi:10.1007/s10637-013-9987-y
28. Grogen PT (2014) Withaferin A: a novel therapeutic approach for malignant brain tumors. PhD Thesis. University of Kansas Medical Center
29. Santagata S, Xu YM, Wijeratne EM et al (2012) Using the heat-shock response to discover anticancer compounds that target protein homeostasis. ACS Chem Biol 7(2):340–349
30. Leeson P (2012) Drug discovery: chemical beauty contest. Nature 481(7382):455–456
31. Banks WA (2009) Characteristics of compounds that cross the blood–brain barrier. BMC Neurol 9(Suppl 1):S3
32. Vaishnavi K, Saxena N, Shah N et al (2012) Differential activities of the two closely related withanolides, Withaferin A and Withanone: bioinformatics and experimental evidences. PLoS One 7:e44419
33. Withaferin A: Safety Data Sheet. Santa Cruz Biotechnology, Inc. http://datasheets.scbt.com/sds/EGHS/EN/sc-200381.pdf
34. Nishikawa Y, Okuzaki D, Fukushima K et al (2015) Withaferin A induces cell death selectively in androgen-independent prostate cancer cells but not in normal fibroblast cells. PLoS One 10(7):e0134137
35. Mandal C, Dutta A, Mallick A et al (2008) Withaferin A induces apoptosis by activating p38 mitogen-activated protein kinase signaling cascade in leukemic cells of lymphoid and myeloid origin through mitochondrial death cascade. Apoptosis 13(12):1450–1464
36. Kakar SS, Ratajczak MZ, Powell KS, Moghadamfalahi M, Miller DM (2014) Withaferin A alone and in combination with cisplatin suppresses growth and metastasis of ovarian cancer by targeting putative cancer stem cells. PLoS One 9(9):e107596
37. Kim SH, Singh SV (2014) Mammary cancer chemoprevention by Withaferin A is accompanied by in vivo suppression of self-renewal of cancer stem cells. Cancer Prev Res 7(7):738–747
38. Lee J, Hahm ER, Marcus AI, Singh SV (2015) Withaferin A inhibits experimental epithelial-mesenchymal transition in MCF-10A cells and suppresses vimentin protein level in vivo in breast tumors. Mol Carcinogen 54(6):417–429
39. Grogan PT, Sarkaria JN, Timmermann BN, Cohen MS (2014) Oxidative cytotoxic agent withaferin A resensitizes temozolomide-resistant glioblastomas via MGMT depletion and induces apoptosis through Akt/mTOR pathway inhibitory modulation. Invest N Drug 32(4):604–617
40. BiotechWeek (2014) Investigators at university of Michigan hospital report findings in apoptosis (oxidative cytotoxic agent withaferin A resensitizes temozolomide-resistant glioblastomas via MGMT depletion and induces apoptosis through Akt/mTOR pathway inhibitory…) (2014) BiotechWeek 442. https://www.highbeam.com/doc/1G1-382765478.html
41. Hahm E, Singh SV (2013) Withaferin A-induced apoptosis in human breast cancer cells is associated with suppression of inhibitor of apoptosis family protein expression. Cancer Lett 334(1):101–108
42. Gasparini G, Weidner N, Maluta S et al (1993) Intratumoral microvessel density and p53 protein: correlation with metastasis in head and neck squamous cell carcinoma. Int J Cancer 55(5):739–744
43. Knizhnik AV, Roos WP, Nikolova T et al (2013) Survival and death strategies in glioma cells: autophagy, senescence and apoptosis triggered by a single type of temozolomide-induced DNA damage. PLoS One 8(1):e55665
44. Huse JT, Holland EC (2010) Targeting brain cancer: advances in the molecular pathology of malignant glioma and medulloblastoma. Nat Rev Cancer 10(5):319–331
45. Wu G, Diaz AK, Paugh BS et al (2014) The genomic landscape of diffuse intrinsic pontine glioma and pediatric non-brainstem high-grade glioma. Nat Genet 46(5):444–450
46. Ohgaki H, Dessen P, Jourde B et al (2004) Genetic pathways to glioblastoma: a population-based study. Cancer Res 64(19):6892–6899
47. Panjamurthy K, Manoharan S, Nirmal MR, Vellaichamy L (2009) Protective role of Withaferin-A on immunoexpression of p53 and bcl-2 in 7,12-dimethylbenz(a)anthracene-induced experimental oral carcinogenesis. Invest N Drug 27(5):447–452
48. Amin R, Karpowicz PA, Carey TE et al (2015) Evasion of anti-growth signaling: a key step in tumorigenesis and potential target for treatment and prophylaxis by natural compounds. Semin Cancer Biol 35:S55–S77. doi: 10.1016/j.semcancer.2015.02.005
49. Kostecka A, Sznarkowska A, Meller K et al (2014) JNK–NQO1 axis drives TAp73-mediated tumor suppression upon oxidative and proteasomal stress. Cell Death Dis 5:e1484

50. Stan SD, Zeng Y, Singh SV (2008) Ayurvedic medicine constituent withaferin a causes G2 and M phase cell cycle arrest in human breast cancer cells. Nutr Cancer 60(Suppl 1):151–160
51. Reifenberger G, Ichimura K, Reifenberger J, Elkahloun AG, Meltzer PS, Collins VP (1996) Refined mapping of 12q13–q15 amplicons in human malignant gliomas suggests CDK4/SAS and MDM2 as independent amplification targets. Cancer Res 56(22):5141–5145
52. Hurley JB, Simon MI, Teplow DB, Robishaw JD, Gilman AG (1984) Homologies between signal transducing G proteins and ras gene products. Science 226(4676):860–862
53. Aizman E, Mor A, Levy A, George J, Kloog Y (2012) Ras inhibition by FTS attenuates brain tumor growth in mice by direct antitumor activity and enhanced reactivity of cytotoxic lymphocytes. Oncotarget 3(2):144–157
54. Samadi AK, Tong X, Mukerji R, Zhang H, Timmermann BN, Cohen MS (2010) Withaferin A, a cytotoxic steroid from *Vassobia breviflora*, induces apoptosis in human head and neck squamous cell carcinoma. J Nat Prod 73(9):1476–1481
55. Krakstad C, Chekenya M (2010) Survival signaling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics. Mol Cancer 9:135
56. Koduru S, Kumar R, Srinivasan S, Evers MB, Damodaran C (2010) Notch-1 inhibition by Withaferin-A: a therapeutic target against colon carcinogenesis. Mol Cancer Ther 9(1):202–210
57. Oh JH, Lee TJ, Kim SH et al (2008) Induction of apoptosis by withaferin A in human leukemia U937 cells through down-regulation of Akt phosphorylation. Apoptosis 13(12):1494–1504
58. Li X, Zhu F, Jiang J et al (2015) Synergistic antitumor activity of withaferin A combined with oxaliplatin triggers reactive oxygen species-mediated inactivation of the PI3K/AKT pathway in human pancreatic cancer cells. Cancer Lett 357(1):219–230
59. Gao Q, Lei T, Ye F (2013) Therapeutic targeting of EGFR-activated metabolic pathways in glioblastoma. Expert Opin Investig Drugs 22(8):1023–1040
60. Lee JJ, Kim BC, Park MJ et al (2011) PTEN status switches cell fate between premature senescence and apoptosis in glioma exposed to ionizing radiation. Cell Death Differ 18(4):666–677
61. Gilmore TD (2006) Introduction to NF-kappaB: players, pathways, perspectives. Oncogene 25(51):6680–6684
62. Karin M (1999) How NFkB is activated: the role of the IkB kinase (IKK) complex. Oncogene 18(49):6867–6874
63. Tergaonkar V (2006) NFkB pathway: a good signaling paradigm and therapeutic target. Int J Biochem Cell Biol 38(10):1647–1653
64. Rayet B, Gelinas C (1999) Aberrant rel/nfkb genes and activity in human cancer. Oncogene 18(49):6938–6947
65. Kaileh M, Berghe WV, Heyerick A et al (2007) Withaferin A strongly elicits IkB kinase b hyperphosphorylation, concomitant with potent inhibition of its kinase activity. J Biol Chem 282(7):4253–4264
66. Yokota Y, Bargagna-Mohan P, Ravindranath PP, Kim KB, Mohan R (2006) Development of withaferin A analogs as probes of angiogenesis. Bioorg Med Chem Lett 16(10):2603–2607
67. Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86(3):353–364
68. Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285(21):1182–1186
69. Reardon DA, Wen PY, Desjardins A, Batchelor TT, Vredenburgh JJ (2008) Glioblastoma multiforme: an emerging paradigm of anti-VEGF therapy. Expert Opin Biol Ther 8(4):541–553
70. Saha S, Islam MK, Shilpi JA, Hasan S (2013) Inhibition of VEGF: a novel mechanism to control angiogenesis by *Withania somnifera's* key metabolite Withaferin A. In Silico Pharmacol 1:11
71. Frumovitz M, Sood AK (2007) Vascular endothelial growth factor (VEGF) pathway as a therapeutic target in gynaecologic malignancies. Gynecol Oncol 104(3):768–778
72. Fack F, Espedal H, Keunen O et al (2015) Bevacizumab treatment induces metabolic adaptation toward anaerobic metabolism in glioblastomas. Acta Neuropathol 129(1):115–131
73. Ebos JM, Kerbel RS (2011) Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. Nat Rev Clin Oncol 8(4):210–221
74. Gao R, Shah N, Lee JS et al (2014) Withanone-rich combination of Ashwagandha withanolides restricts metastasis and angiogenesis through hnRNP-K. Mol Cancer Ther 12:2930–2940
75. Mohan R, Hammers HJ, Bargagna-Mohan P et al (2004) Withaferin A is a potent inhibitor of angiogenesis. Angiogenesis 7(2):115–122
76. Bargagna-Mohan P, Hamza A, Kim Y et al (2007) The tumor Inhibitor and antiangiogenic agent Withaferin A targets the intermediate filament protein vimentin. Chem Biol 14(6): 623–634
77. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674
78. Grogan PT, Sleder KD, Samadi AK, Zhang H, Timmermann BN, Cohen MS (2013) Cytotoxicity of withaferin A in glioblastomas involves induction of an oxidative stress-mediated heat shock response while altering Akt/mTOR and MAPK signaling pathways. Invest N Drug 31(3):545–557
79. Shah N, Kataria H, Kaul SC, Ishii T, Kaur G, Wadhwa R (2009) Effect of the alcoholic extract of Ashwagandha leaves and its components on proliferation, migration, and differentiation of glioblastoma cells: combinational approach for enhanced differentiation. Cancer Sci 100(9):1740–1747