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By

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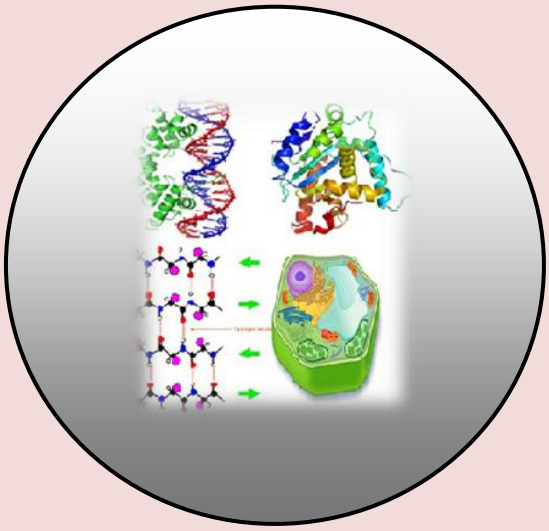

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Multifunction Activities of withaferin A and Comprehensive Assessment of the Gene Involved in its Biosynthesis from *Withania somnifera*: In Molecular Insight

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ABSTRACT

Withania somnifera (WS), is an important medicinal plant used in Ayurvedic system, it is a rich source of pharmaceutically active secondary metabolites known as withanolides. In addition to withanolides, Withaferin-A, withanolide-A, withanoside-IV and some sitoindosides are the major constituents of WS. Among them, the most bioactive compound is Withaferin A that has anti-inflammatory, anti-invasive and anti-angiogenic property. In perspective of modern pharmacology, there is a need to understand the underlying mechanism of the broad range of bioactivities exerted by Withaferin A. Therefore, a lot of effort was made to explore the role of Withaferin A intracellulary and characterize its target proteins. Withanolides are synthesised by mevalonate (MVA) pathway and methyl-D-erythritol-4-phosphate (MEP) pathway involving metabolic intermediacy of 24-methylene (C30-terpenoid) cholesterol. Several putative genes encode enzymes for biosynthesis of terpenoid backbone. In this review we will explain the molecular basis of the health-promoting potential of Withaferin A and explore the gene involved in the biosynthesis of withanolides and this study will leads to elucidating the neuroprotective property of Withaferin A and withanolide biosynthesis and development of new tools for functional genomics of this important medicinal plant.

Keywords: Antioxidant, Withaferin A, salicylic acid, withanolide, terpenoid, secondary metabolites.

INTRODUCTION

Medicinal plant is the boon for biotechnology and the researcher, most of the drug industry also dependent on the plants for the production of their pharmaceutical compounds. In the developing and the developed countries the demand on the plant based therapies increases due to the growing credit that they are natural products, being non-narcotic, having no side-effects, easily available at affordable prices and sometimes the only source of health care available to the poor. *Withania somnifera* (L) Dunal, is a member of the Solanaceae family commonly known as Ashwagandha most widely used herb in Ayurvedic and indigenous medical system for over 3000 years. First reports observed that the tuber or root is the most important part used in medicine, and it is rich in alkaloid (Alam et al., 2012) and lactones called withanolides. It is a valuable constituent in traditional Ayurvedic drug preparations against many diseases such as hiccup, female disorders, cough, rheumatism and dropsy (Orru et al., 2013).

Withania somnifera: Ashwagandha

The Solanaceae family is comprised of 84 genera that include about 3,000 species, scattered throughout the world. The sixty five known *Withania* species are widely distributed in the drier parts of tropical and subtropical regions of Punjab, Haryana, UttarPradesh, Uttarakhand, Bihar, Jharkhand, Rajasthan, Madhya Pradesh, Maharastra and some parts of Himachal Pradesh and Jammu and Kashmir ascending up to 1,650m in the Himalayas. Among them, only two species, *Withania somnifera* (Figure 1) and *Withania coagulans*, are economically and medicinally significant, being used and cultivated in several regions such as Pakistan, Afghanistan, Palestine, Egypt, Jordan, Morocco, Spain, Canary Island, Eastern Africa, Congo, Madagascar, South Africa and India (Bhattacharya et al., 2001; Grandhi et al., 1994).

Bioactive compounds present in *W. somnifera*

Withania somnifera contains many important chemical constituents. About 35 chemical constituents have been identified, extracted, and isolated. Among these the chemistry of *W. somnifera* has been extensively studied. Amorphous alkaloid (C12H16N2) was isolated from a South African strain of *W. somnifera,* its Chemical characterization was done. Later, investigated a plant from Bengal (India) was investigated & it confirmed the presence of the alkaloid and they reported the presence of nicotine and seven other alkaloids from the roots which they named (without structural information) as somniferinine, withanaminine, somniferine, withamine, pseudowithamine, withanmine and somnine, (Dhuley et al., 1997). There are several reports which shows the presence of other alkaloids such as anaferine (bis (2-piperidylmethyl) ketone); pseudotropine;isopelletierine; tropine; 3-tropyltigloate; 3α-tigloyloxtropine; cuscohygrine; dlisopelletierine; hygrine; mesoanaferine; anahygrine; choline; withanine; withananinevisamine, withasomnine pseudowithanine, hentriacontane, somniferine; and ashwagandhine in *W. somnifera* (Chaurasia et al.1980). The plant also contain chemical constituents like withaniol, acylsteryl glucosides, starch, hantreacotane, ducitol, reducing sugar, a variety of amino acids including, tryptophan, proline, tyrosine, alanine, aspartic acid glycine, , cystine, glutamic acid, and high amount of iron. The presence of catechin was confirmed in *W. somnifera* by analysis of high performance liquid chromatography (HPLC).

At present, more than 12 alkaloids, 40 withanolides, several sitoindosides, and which is also a withanolide having a glucose molecule at carbon 27 have been isolated and reported from several parts *Withania* species such as aerial parts, roots and berries. The concentration of withanolides usually ranges from 0.001 to 0.5% dry weight (Alam et al*.,* 2012).

Table 1. List of publications demonstrating in vitro activities of WA in a wide variety of cell types including inhibition of NF-kB activation by different stimuli, induction of cell death, inhibition of angiogenesis and inhibition of proliferation.

NF-KB INHIBITING ACTIVITY

CELL LINE	INDUCER	GENES AFFECTED
Macrophage cell line (RAW 264.7)	LPS	iNOS
Leukemia cell line (U937)	TNF	
Peripheral blood and synovial fluid mononuclear cells	LPS	TNFa, IL1b, IL12 p40
Islet cells (diabetes)	Cytokine mixture (IL1+ TNF+ IFNg)	iNOS, IL1b, RANTES, IP10
Erythroleukemic cell line (K562) Fibrosarcoma (L929)	TNF, PMA	IL6, IL8, A1,MCP1, A20, cyclinD1, VEGF, MDR1
Multiple myeloma cell line (U266)	Constitutive	
Breast carcinoma (MDA-MB231)	Constitutive	IL6
Colon carcinoma cell lines (HCT116, SW480, SW620)	Constitutive	
Lung epithelial cell line (A549)	TNF	ICAM, VCAM
Immortalized Cystic Fibrosis airway cell line (KKLEB),	*Psuodomonas*	IL8
Embryonic kidney (HEK)	*Aeruginosa* filtrate, TNF	
Prostate cancer cells (PC-3)	Constitutive	
Fibrosarcoma (L929)	TNF	IL6, RANTES, IkBa
Myeloid leukemia (KBM-5)	TNF	
Endothelial cells (HUVEC)	TNF, LPS	
T-cell lymphoma (HUT-78) Promyelocytic leulemia cells (HL60)	Constitutive	
PMBC	LPS	
Spinal cord tissue	Overexpression of TAR–DNA binding protein TDP-43	
Cervical cancer cell line (Hela)		
Endothelial cells (HUVEC)	HMGB1	IL6, TNFα
Colorectal adenocarcinoma cell line (SW480)	MDP	

APOPTOSIS INDUCING ACTIVITY

CELL TYPE	PATHWAY
Leukemic cell lines and lymphoblastic and myeloid leukemia	Apoptosis via mitochondrial death cascade
Erythroeukemic cell line	Caspase dependent apoptosis
Cholangiocarcinoma celllines	Apoptosis correlated with upregulation of tumor suppressor PAR-4
Prostate cancer cells	PAR-4 dependent apoptosis
Human leukemia	Caspase-dependent apoptosis by activationof JNK and inhibition of Akt
Lung cancer cell line	
T cell leukemia cell line	Apoptosis correlating with LXRa activation
Thyroid cancer cell line	Caspase-dependent apoptosis
Androgen dependent prostate cencer	Proteasome/caspase-dependent apoptosis
Murine embryonic fibroblast cell line	Vimentin dependent cell death
Renal carcinoma cell line	ER-stress dependent apoptosis
Myeloid leukemia cell line	Apoptosis via ROS and mitochondrial death cascade
Breast carcinoma cell line	FOXO3A dependent apoptosis
Breast cancer cell line	Caspase dependent apoptosis
Breat cancer cell line	ROS, p53 and ERa dependent apoptosis
Cutaneous melanoma cell line	Caspase and ROS dependent apoptosis with down-regulation of Bcl2
Cervical cancer cell line	Down regulation of human papilloma virus oncogenes
Renal cancer cell line	ROS mediated apoptosis
Ovarian carcinoma cells	Apoptosis
Pancreatic cancer cell line	Apoptosis
Cervix carcinoma cell line	Apoptosis
Breast cancer cell lines	Caspase dependent apoptosis correlating with hsp90 inhibition

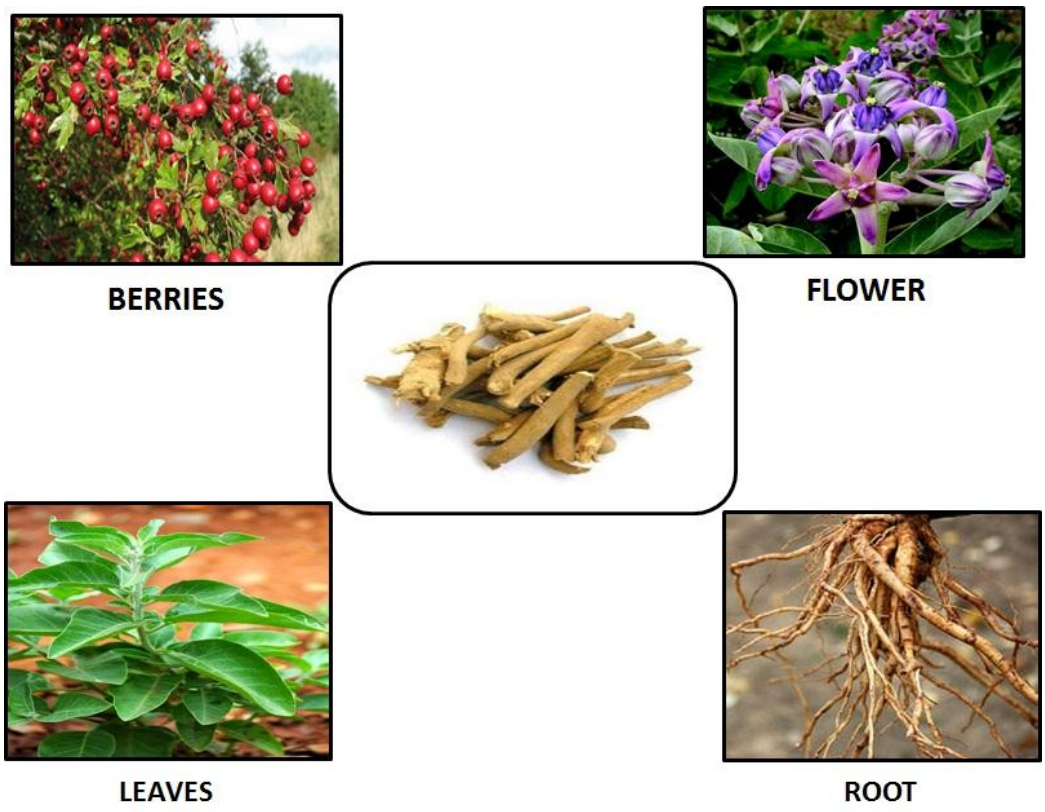

Figure 1.

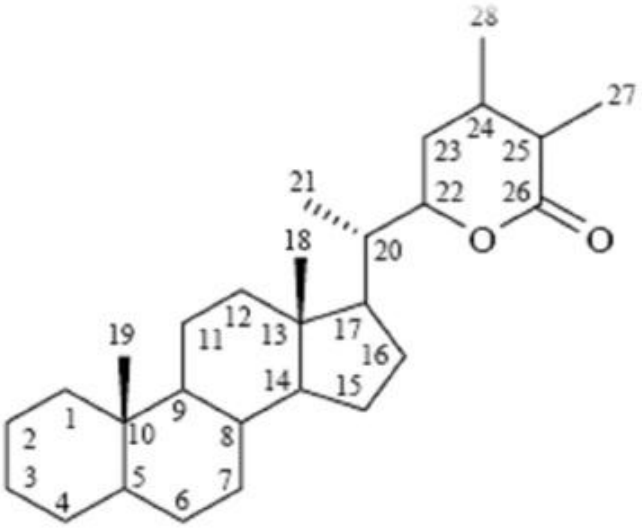

Figure 2.

Withanolide Structure

Withanolides are generally defined as C-28 steroidal lactones which contains lactone ring with C-22 and C-26 oxygen that helps to form a six or five membered lactone ring on an Ergostane skeleton, intact ergostane or rearranged, constitutes the basic structure (Figure 2) of all withanolides (Glotter et al., 1991). The withanolides skeleton may be defined as 22-hydroxy ergostane-26-oic acid-26, 22-olide. The novel structural variants of withanolides are formed by the modifications of carboxylic skeleton and side chains and it is described as modified withanolides or ergostane-type steroids related to withanolides (Kirson et al., 1981)

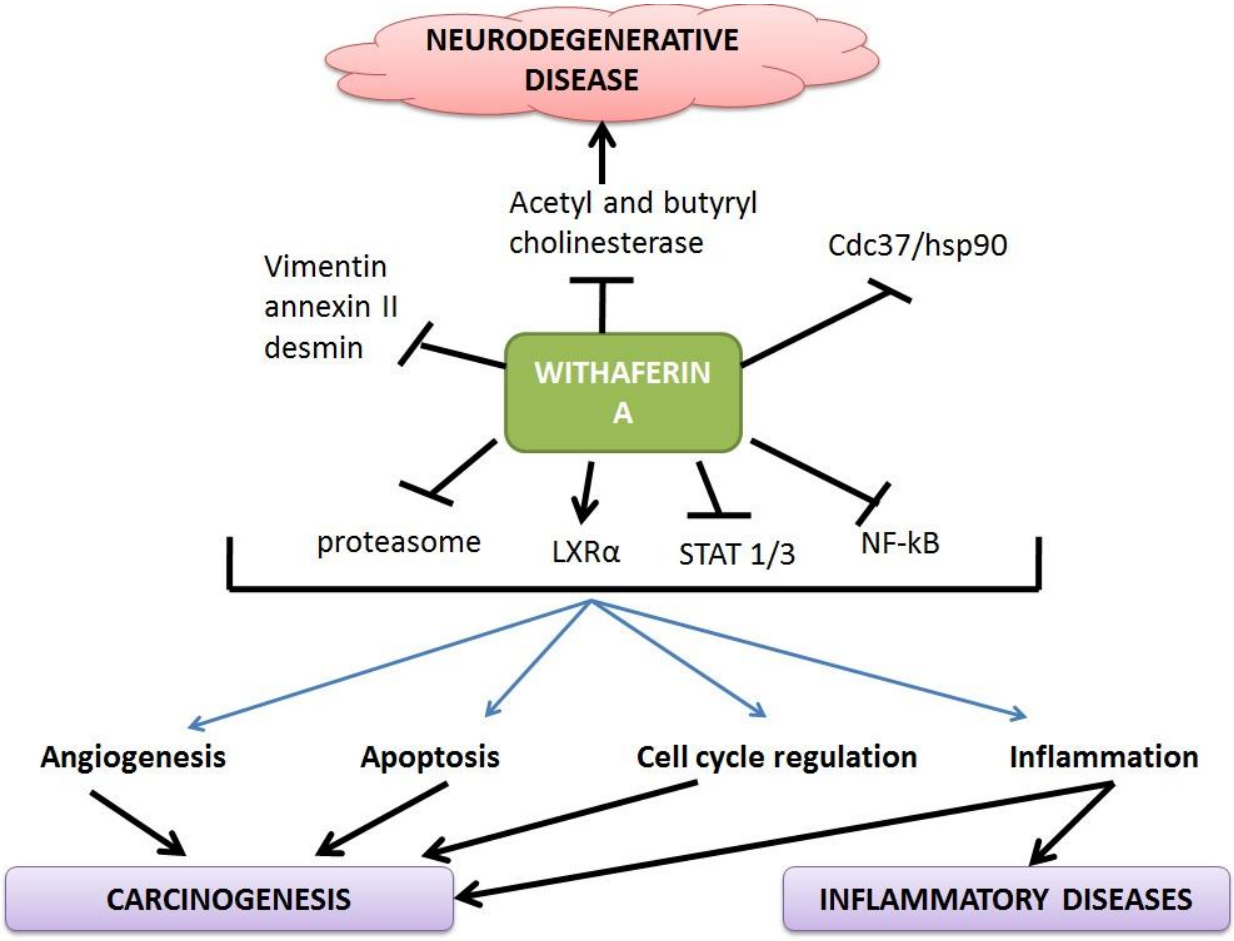

Figure 3.

Withaferin A (WA)

Withaferin A (4β, 27-dihydroxy-1-oxo-5β, 6β-epoxywitha-2-24-dienolide) was the first member of Withanolides of compounds to be isolated from *W. somnifera*. The interesting biological activity of this compound led to a thorough chemical investigation of the plant and numerous compounds with similar structural features were isolated (Glotter, 1991).

Withaferin A has been extensively studied for their biological activities (Table 1). Steroidal part of withanolide contain a specific arrangement of four cycloalkane ring structures, three cyclohexane rings and one cyclopentane ring that are joined to each other and the lactone part is a cyclic ester which in case of WA is characterized by a closed ring consisting of 5 carbon atoms and a single oxygen atom. The biological activities of WA is due to the epoxy functional group at C-5,6 which is indicated by structure–function analysis of several withanolides. Misra et al., 2008). (Santagata et al., 2011, Kaileh et al., 2007, Suttana et al., 2010; Falsey et al., 2006; Bargagna-Mohan et al., 2010; Yokota et al., 2006).

Molecular targets of Withaferin A

Several studies demonstrated the anti-inflammatory, pro-apoptotic anti-proliferative effects of WA. How WA can exert all these activities remains largely enigmatic? WA can directly interact with several protein and change its activity, have already been identified. These interactions can further influence the activity of secondary targets and related signal transduction pathways. Among the direct targets of WA structural proteins, proteases, transcription factors as well as kinases have been characterized which will be described in this review in more detail (Figure 3).

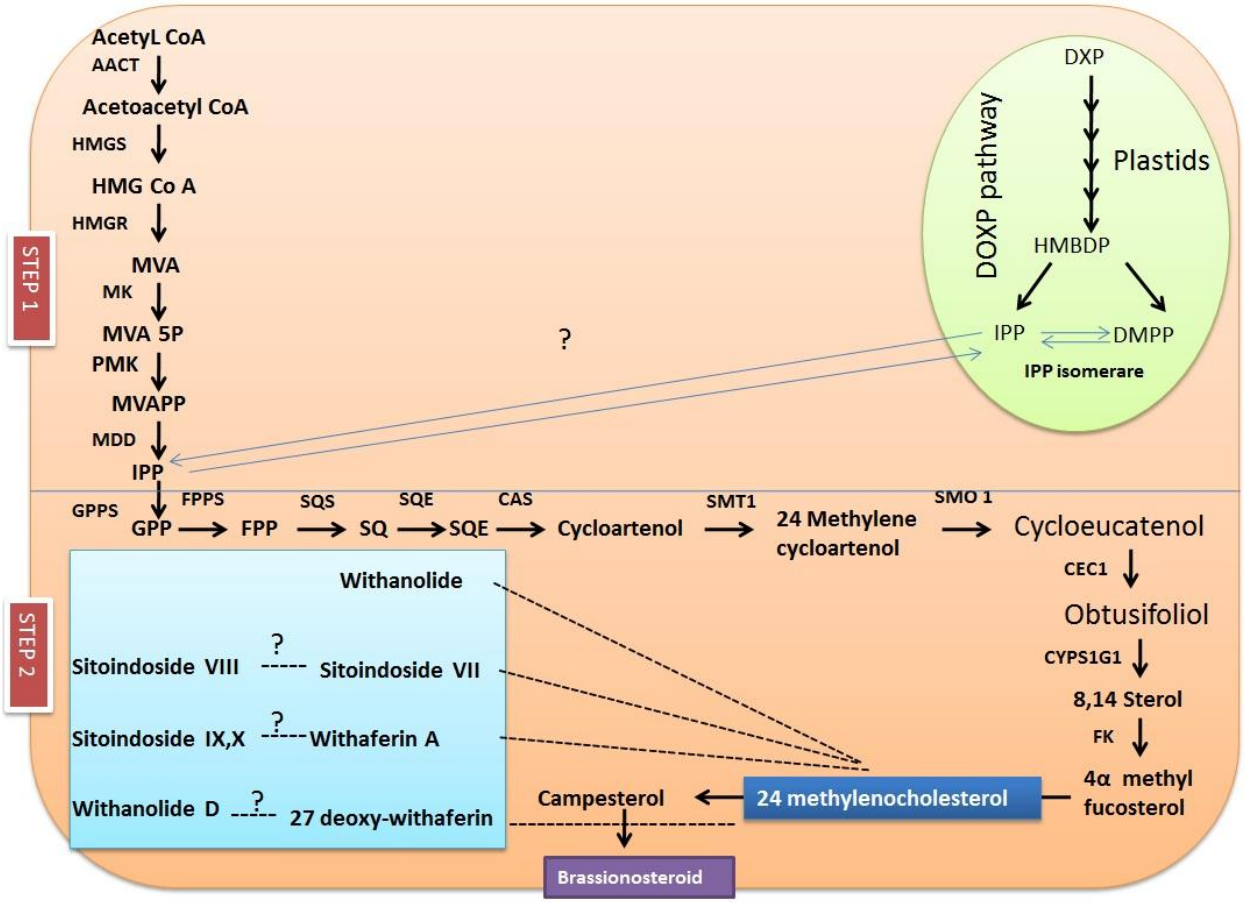

Figure 4.

Targeting the Proteasome Complex by WA

One of the targets of WA to undergo covalent interaction is the proteasome, the protease complex of the ubiquitin and proteasome dependent proteolytic system (UPS), which is the major eukaryotic pathway for regulated protein degradation. Initially, UPS pathway marks the substrates for degradation by the attachment of multiple ubiquitin moieties. This process involves 3 sequential steps and 3 different enzyme are involves in this steps: first activation, second conjugation and third, ligation of the 76 amino acids long ubiquitin protein to a lysine residue of the target protein. Attachment of multiple ubiquitin entities leading to formation of a K48-linked poly-ubiquitin chain which establishes a signal for degradation by the proteasome. This highly selective proteinase complex is composed of a cylindrical 20S core particle and two 19S cap particles docking at both ends of the 20S core unit. 3 different proteolytic activities are mediated by 3 b-subunits of the 20S core: a caspase-like activity by b1, a trypsin like by b2 and a chymotrypsin like by b5. The chymotrypsin like activity was already shown to be inhibited by dietary flavonoids containing aromatic ketone structures (Chen et al., 2005). Yang and colleagues demonstrated that WA also inhibits this chymotrypsin like activity in a cell free assay system as well as in the androgenin dependent PC3 prostate cancer cells at high dose. Also in silico docking studies model the orientation and conformation of WA allowing nucleophilic attack with the proteins, further supporting the hypothesis of covalent linkage (Yang et al., 2007). Molecular docking results suggest binding of WA to the catalytically active N-terminal Thr1 residue of the b5 subunit (Yang et al., 2007; Grover et al., 2010). In contrast to the previously described reactive carbon sites in WA, this chemical structure analysis rather pinpoints the C1 and C14 atoms of the steroidal structure as the highly susceptible reactive sites. In a dose dependent and time dependent manner the proteasome inhibiting effect of WA leads to accumulation of ubiquitinated proteins and increased expression of ubiquitination-degradation sensitive proteins (Yokota et al., 2006; Mohan et al., 2004; Yang et al., 2007). However, an independent in vitro study indicated only slight inhibition of proteasomal activity by WA, in comparison with the well-known proteasome inhibitor epoxomycin (Bargagna-Mohan et al., 2007). Strikingly, treatment of breast carcinoma cells with WA induced proteasome- dependent downregulation of Estrogen Receptor a which could be counteracted by co-treatment with the proteasome inhibitor MG132 (Zhang et al., 2011), seemingly countering the proteasome inhibiting function of WA. Conceivably different protease activities of the proteasome are involved so emphasizing the necessity of more specified analysis of proteasomal degradation. The UPS pathway has multiple essential biological roles and often its malfunctioning is related to various human diseases including different cancer types, cardiovascular and neurodegenerative diseases. Therefore, the ubiquitin–proteasome pathway is widely recognized as an important target for drug discovery, because many important processes such as activation of the proinflammatory transcription factor NF-kB as well as apoptosis are orchestrated through the orderly degradation of key regulatory proteins. Interestingly, the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals) has been approved by the US FDA for treatment of multiple myeloma and relapsed mantle cell lymphoma. So targeting the ubiquitin proteolytic processing might be a major step by which WA can exert its distinct anti-tumor and anti-inflammatory pharmacological activities.

Regulation of the Transcription Factor NF-kB by WA

Though the above stated crucial role of ubiquitination and proteasomal degradation in the signal transduction pathway leading to activation of the transcription factor NF-kB. NF-kB is involved in the upregulation of proteins that promote cell survival, stimulate growth, induce angiogenesis and reduce susceptibility to apoptosis. Regarding the necessary control of expression of these NF-kB driven proteins, activation of NF-kB is tightly regulated. Under quiescent conditions, NF-kB binds to IkB (cytoplasmic retention protein) inhibitor in cytoplasm which masks its nuclear localization signals. Several inflammatory stimuli, including pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF) and Interleukin-1 (IL1) and also infection with microbial pathogens, rapidly activate NF-kB, by triggering of the specific cognate receptors. This event induces initiation of receptor specific signal transduction pathways.

The NF-kB inhibiting capacity of WA has been studied in several different cell types after triggering by different stimuli. NF-kB regulated gene expression is strongly altered in these cells. It is also reported that continuously active NF-kB can be blocked by WA in different cell lines which might be important for treatment of several hematologic cancers including acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and multiple myeloma (MM), in which constitutively active NF-kB has been observed. The increase in activity of NF-kB has also been observed in several chronic inflammatory diseases such as asthma, Crohn's disease, rheumatoid arthritis, as well as in metabolic disorders including obesity, type 2 diabetes, and atherosclerosis. Since it has the central role in several diseases via coordination of inflammatory as well as anti-apoptotic responses, a lot of effort has been put in the search and development of new therapeutic products exerting NF-kB inhibiting potential. WA was found to be a potent candidate for the treatment of gouty arthritis and chronic proliferative arthritis in animal models (Kaileh et al., 2007; Maitra et al., 2009; Sabina et al., 2008).

Therapeutic Implications of Multi-target Actions of WA

In the previous section of this review, we have described that WA possess anti-inflammatory, pro-apoptotic, anti-angiogenic and anti-proliferative activities. The combined effect of these activities makes WA a potential drug candidate for treatment of different types of cancer and other diseases. Indeed initial therapeutic trials in rodent animal models give promising results for the treatment of different cancer types including medullary thyroid cancer (Samadi et al., 2010); breast cancer (Thaiparambil et al., 2011); pancreatic cancer (Yu et al., 2010), cervical cancer (Mungala et al., 2011), orthotopic glioma (Santagata et al., 2011), lung cancer (Gupta et al., 2012), leiomyosarcoma and fibrosarcoma (Lahat et al., 2010). Also a chemopreventive potential can be attributed to WA as evaluated in animal models of polynuclear aromatic hydrocarbon dimethylebenz(a)anthracene (DMBA) induced tumorigenesis (Manoharan et al., 2009; Panjamurthy et al., 2008; Panjamurthy et al., 2009). So WA exerts clear therapeutic effects, even though rapid clearance of WA was observed in the plasma. In normal tissues the therapeutic dose of WA shows only a minimal toxicity (Thaiparambil et al., 2011). Also in vitro, the pro-apoptotic activity of WA was shown to be specific for cancer cells, whereas it did not show any respond for normal peripheral blood mononuclear cell derived lymphocytes and monocytes (Mandal et al., 2008; Mehrotra et al., 2011).

In vitro normal human fibroblast also responded to WA but only at higher doses (Widodo et al., 2009; Samadi et al., 2009; Widodo et al., 2010). The selectivity of WA in killing abnormal cells is not clarified and requires more in depth investigation. Carcinogenesis is a multistage process which is characterized by deregulation of multiple biochemical and physiological pathways controlling cell growth, survival and apoptosis. In this regard targeting multiple signaling molecules that collectively contribute to tumor development or progression can be definitely advantageous. The chemosensitizing and radiosensitizing effects of WA have been evaluated in vitro as well as in vivo and both indicated that combination therapy enhances significantly the therapeutic efficiency (Devi et al., 1996; Devi et al., 2003; Yang et al., 2011). However care should be taken since these radiosensitizing activities of WA seem to be less tumor specific as increased toxicity of normal bone marrow cells could be observed (Gana soundari et al., 1997). Further studies are required to investigate the extensiveness of this observation under conditions of localized application of radiotherapy instead of whole body treatment.

Genes involved in withanolide Biosynthesis

Withanolides are C28-steroidal lactones having a triterpenoidal metabolic origin synthesized via mevalonate (MVA) pathway and methyl-D-erythritol-4-phosphate (MEP) pathway involving metabolic intermediacy of 24-methylene (C30-terpenoid) cholesterol.

Subcellular localization studies suggest that both paralogs of sterol Δ-7 reductase (WsDWF5-1 and WsDWF5-2) are localized in the endoplasmic reticulum (ER) thus supporting their indispensible role in withanolide biosynthesis. These secondary plant products biosynthesis and accumulation is species as well as chemotype specific and under tight spatial and temporal regulation of gene expression (Gupta et al. 2013a, 2015; Pathak et al. 2013), which limit their proper industrial utilization and drug development. Although biotechnological approaches have been successful in enhancing biosynthesis of few valuable secondary metabolites (Pandey et al. 2014, 2015; Zhang et al. 2015), these approaches cannot be used for other diverse molecules due to several technical bottlenecks and the lack of understanding about their synthesis and regulation at different steps (Sivanandhan et al., 2014).

Biosynthesis of withanolide uses isoprene units (isopentenylnpyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP)) as precursors. Isoprene synthesis or isoprenogenesis includes two autonomous pathways, the classical cytosolic mevalonate (MVA) pathway and an alternative plastid localized 2-C-methyl- D-erythritol-4-phosphate (MEP) pathway which lead to biosynthesis of 24-methylene cholesterol (C30 terpenoid) (Bhat et al. 2012).

Several genes encode full-length polypeptides that are putatively involved in various intermediate steps of withanolide biosynthesis. This suggests that some of enzymes involved in intermediate steps are encoded by multigene families.

Modulation in the expression of selected genes in response to exogenous applications of SA and MJ was assessed considering early (up to 6 h post-treatment) and late (from 24 to 72 h post-treatment) responses. In general, expression levels of most of the genes followed a similar pattern to both the treatments and did not exhibit significant modulation during early response.

However, up to sixfold gradual enhancement in expression was observed during early response for some of the steps 1 and 2 genes including WsCDPMEK, WsHMGR3, WsFPPS1, WsFK and WsHYD1 in response to MJ. SA treatment showed a biphasic pattern during early response in some of the genes (WsCDPMEK, WsHMGR3, WsIPI, WsFPPS1, WsFPPS2 and WsDWF5-2) with an impulsive enhancement in expression during the first 3 h whichwas reverted back in the next 3 h post-treatment. A gradual decrease in the expression was observed in case of WsDXS1 for both, SA and MJ, treatments, whereas similar transition was observed in case of WsDWF5-1 for SA treatment (Figs. 6 and 7). Most of the genes displayed significant modulation in their expression levels, rendering a biphasic fashion, during the late response towards both the elicitors. Expression levels of most of the genes were significantly lower as compared to early response phase. Some of the genes (WsHMGR3, WsMK, WsDXS1, WsDXS2, WsCDPMEK, WsIPI, WsFPPS1, WsFPPS2, WsHYD1 and WsDWF5-2) showed upregulation in their expression at 24 h post treatment followed by downregulation. Interestingly, few genes (WsHMGR2, WsDXR and WsCAS) showed a contrary expression pattern in response to elicitor treatments. WsHMGR1 showed maximum upregulation, 6-fold in response to MJ and 45-fold in response to SA at 48 and 72 h post-treatment, respectively. WsSMO1 showed a markedly upregulated expression, (45- and 15-fold, at 24 h for SA and MJ treatments, respectively). Expression of WsFK and WsDWF5-1 decreased gradually in the late response phase for both the elicitor treatments (Figure 4). Majority of the genes involved in the intermediate steps of the biosynthesis of triterpenoid as well as putatively involved in withanolide biosynthesis have been characterized from *W. somnifera* opening up a broader horizon towards exploration of biosynthesis of specialized molecules in this important medicinal plant. Based on transcriptomic analysis, 18 terpenoid biosynthesis-related genes were selected, which can be grouped into nine single- and four multi-gene families. Using a comprehensive approach, phylogenetic relationship and gene expression patterns were analyzed in different tissues and upon elicitor treatments. Some of the key enzymes are encoded by multiple genes such as WsDXS, WsHMGR, WsFPPS and WsDWF5 with different expression patterns reflecting complexity of terpenoid biosynthesis in *W. somnifera.* Our studies remain limited due to lack of molecular knowledge regarding regulation of these genes and therefore a high throughput genomic analysis of the plant as well as functional genomics could help in further understanding of withanolide biosynthesis in *W. somnifera*. Such studies would not only be useful for understanding the withanolide biosynthesis but will also provide molecular wealth for biotechnological improvement of this medicinal plant.

CONCLUSIONS

Although the mechanisms of action of most natural medicinal products are largely unclear, these compounds have already become more and more important in drug discovery for the treatment of various human diseases. These compounds also became the subject of several research projects since increasing the knowledge of the molecular mechanistic evidences of these phytochemicals can improve the accuracy of their therapeutic use. Also for the W. somnifera derived withanolide, WA, our gain in knowledge is increasing over the last years as reflected in increased output of publications.

Though several molecular targets ofWA have already been identified, which partially can explain the broad range of in vivo biological effects of WA, major concerns on the therapeutic use of WA still persist. While the therapeutic potential of WA seems very promising in studies using animal models, to our knowledge, the therapeutic, pharmacological and toxicological properties of Withaferin A in human have not intensively been investigated. So care should be taken and intensive pre-clinical studies are absolutely required to determine a safe drug dose for administration and to justify clinical trials for the further evaluation of the efficacy of WA for the treatment of a wide range of diseases.

Conflicts of interest

We declare that none of the authors have financial interest related to this work.

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