Chapter 14
Natural Withanolides in the Treatment of Chronic Diseases

Peter T. White, Chitra Subramanian, Hashim F. Motiwala and Mark S. Cohen

Abstract Withanolides, and in particular extracts from *Withania somnifera*, have been used for over 3,000 years in traditional Ayurvedic and Unani Indian medical systems as well as within several other Asian countries. Traditionally, the extracts were ascribed a wide range of pharmacologic properties with corresponding medical uses, including adaptogenic, diuretic, anti-inflammatory, sedative/anxiolytic, cytotoxic, antitussive, and immunomodulatory. Since the discovery of the archetype withaferin A in 1965, approximately 900 of these naturally occurring, polyoxygenated steroidal lactones with 28-carbon ergostane skeletons have been discovered across 24 diverse structural types. Subsequently, extensive pharmacologic research has identified multiple mechanisms of action across key inflammatory pathways. In this chapter we identify and describe the major withanolides with anti-inflammatory properties, illustrate their role within essential and supportive inflammatory pathways (including NF-κB, JAK/STAT, AP-1, PPARγ, Hsp90 Nrf2, and HIF-1), and then discuss the clinical application of these withanolides in inflammation-mediated chronic diseases (including arthritis, autoimmune, cancer, neurodegenerative, and neurobehavioral). These naturally derived compounds exhibit remarkable biologic activity across these complex disease processes, while showing minimal adverse effects. As novel compounds and analogs continue to be discovered, characterized, and clinically evaluated, the interest in withanolides as a novel therapeutic only continues to grow.

Keywords Autoimmune · Cancer · Inflammation · Neurodegenerative · NF-κB · Withaferin A · Withanolide

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Abbreviations

5XFAD	5 FAD mutations carried on APP and PS1 transgenes
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADAM10	Adisintegrin and metalloproteinase domain-containing protein 10, or α-secretase
AP-1	Activator protein 1
APP	Amyloid precursor protein
BACE	APP cleaving enzyme 1 or β-secretase
BChE	Butyrylcholinesterase
Bfl-1/A1	Bcl-2-related protein A1
C/EBPα	CCAAT/enhancer-binding proteinα
CCR7	Chemokine (C–C motif) receptor 7
cFLIP	C-FADD-like IL-1β-converting enzyme–inhibitory proteins
CNS	Central nervous system
COX	Cyclooxygenase
CSC	Cancer stem cell
EGF	Epidermal growth factor
EGFR	EGF receptor
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
GABA	Gamma-aminobutyric acid
GMP	Good manufacturing process
HD	Huntington's disease
HIF-1	Hypoxia inducible factor-1
HMGB1	High mobility group box 1
hnRNP-K	Heterogeneous nuclear ribonucleoprotein
KHPA	Hypothalamic–pituitary–adrenal
Hsp	Heat shock protein
HTT	Mutant Huntingtin
IAP1	Inhibitor of apoptosis protein-1
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IFN	Interferon
IKK	I kappa B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK1	c-Jun N-terminal protein kinase
Keap1	Kelch like ECH-associated protein-1
LPS	Lipopolysaccharide

LTB_4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MCE	Mitotic clonal expansion,
MCP-1	Monocyte chemoattractant protein-1
MMPs	Matrix metalloproteinases
MMTV-neu	Mouse mammary tumor virus-neu
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Mass spectrometry
mTOR	Mechanistic target of rapamycin
MUC-1	Mucin 1
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
OCD	Obsessive-compulsive disorder
PBMC	Peripheral blood mononuclear cells
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E2
PI3K	Phosphatidylinositol-3-kinase
PPARs	Peroxisome proliferator-activated receptors
QR	Quinone reductase
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
SAR	Structural–activity relationship
SFMC	Synovial fluid mononuclear cells
SOD	Super oxide dismutase
STAT	Signal transducer and activator of transcription
TAK1	Transforming growth factor-β-activating kinase
TGF-β	Transforming growth factor beta
Th	T-helper
TLRs	Toll-like receptors
TNBS	Trinitrobenzyl sulfonic acid
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TWIST	T*wist* family BHLH transcription factor
UPLC	Ultra-performance liquid chromatography
VEGF	Vascular endothelial growth factor
WA	Withaferin A
WS	*Withania somnifera*

14.1 Introduction

Withanolides are a group of naturally occurring polyoxygenated steroidal lactones assembled on a C_{28} ergostane skeleton. The structural skeleton of withanolides usually varies in the nature and number of oxygenated substituents and the degree of unsaturation of the rings. Structurally diverse withanolides are typically classified based on the arrangement of the C-17 side chain into a major C-22/C-26 δ-lactone/lactol group and a minor C-23/C-26 γ-lactone/lactol group with few exceptions and about 90 % of these compounds possess ketone functionality at C-1 (Figs. 14.1, 14.2) [1–4]. Additionally, withanolides with a C-17 δ-lactone side chain, as shown in Fig. 14.1, can be further categorized into withanolides with an unmodified skeleton (e.g., withaferin A and withaperuvin B) and into those with a

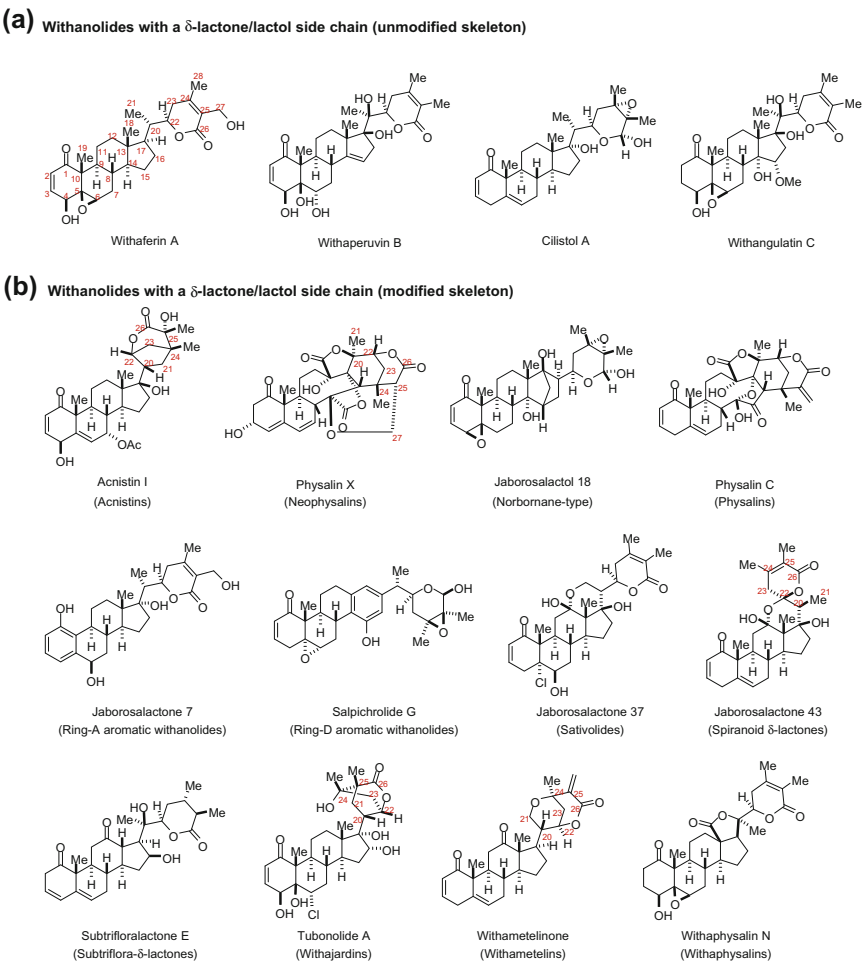


Fig. 14.1 Withanolides with a δ-lactone ring

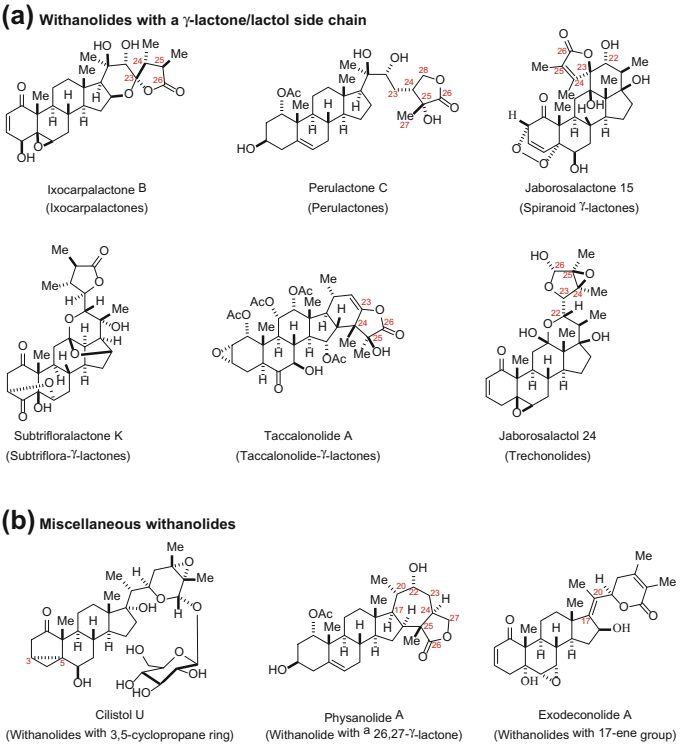


Fig. 14.2 Withanolides with a γ-lactone ring and unclassified structural type

modified skeleton (e.g., physalin C and withametelinone) [1, 2, 4]. The occurrence of unmodified withanolides is more common in nature with approximately 580 of these naturally occurring withanolides reported in the family Solanaceae alone [1–3]. Structurally more complex withanolides with a modified skeleton both in the steroid nucleus and the side chain could possibly result from the biogenetic transformations of unmodified withanolides [3, 5].

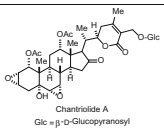
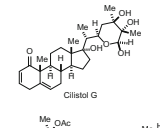
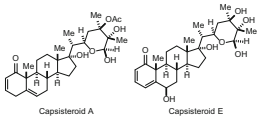
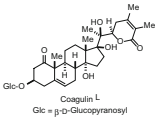
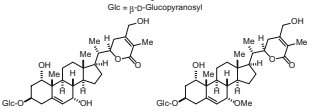
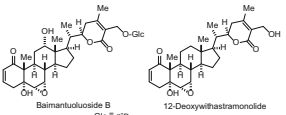
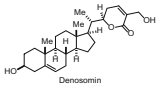
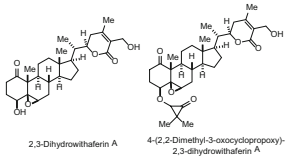
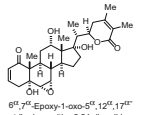
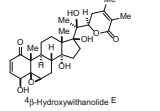
Withaferin A (WA), an archetype of this class was discovered from *Withania somnifera* (WS) or Ashwagandha in 1965 [6]. In the past 50 years, approximately 900 withanolides falling into 24 diverse structural types have been discovered [1, 3]. Withanolides are mainly distributed in various genera of Solanaceae, which includes Acnistus, Aureliana, Brachistus, Browallia, Datura, Deprea, Discopodium, Dunalia, Exodeconus, Hyoscyamus, Iochroma, Jaborosa, Larnax, Lycium, Mandragora, Nicandra, Physalis, Salpichroa, Saracha, Solanum, Trechonaetes, Tubocapsicum, Vassobia, Withania, and Witheringia [1–3, 7–9]. A minor population of withanolides has been isolated from other plant sources such as Dioscoreaceae, Fabaceae, Labiatae, Lamiaceae, Leguminosae, Myrtaceae, and Taccaceae [1–3] and interestingly from marine sources of Alcyoniidae family [10–12].

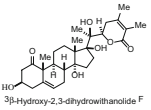
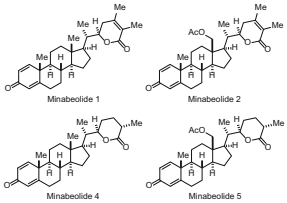
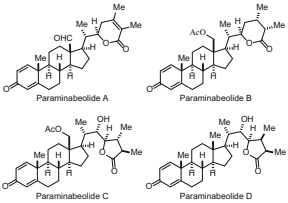
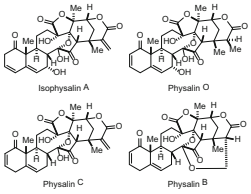
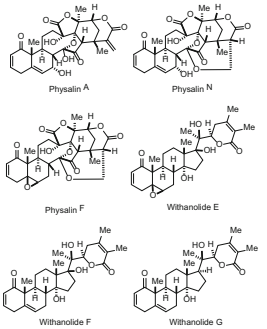
Structurally varied withanolides have received significant attention due to their versatile biological activities demonstrated in vitro and/or in vivo. These activities have been described as antitumor [7, 13–15], cytotoxic [16–20], apoptotic [21–23], anti-inflammatory [9, 10, 24–31], immunomodulating [32–34], antimicrobial [35–37], antistress [34], antioxidant [38], anti-neurodegenerative [39–41], radiosensitizing [42, 43], and insect antifeedant [44, 45]. Withaferin A, the most studied withanolide, possesses a wide array of the pharmacological activities described above and thus carries a great clinical potential for drug development [1, 4, 46–51]. Most notably, the antitumor and associated anti-inflammatory activities of WA and other withanolides results from targeting multiple signaling pathways simultaneously, particularly the nuclear factor kappa B (NF-κB), signal transducer and activator of transcription (STAT), and ubiquitin proteasome pathways (see Tables 14.1, 14.2) [52–57]. The potent biological activities of withanolides such as WA and tubocapsenolide A, especially the antitumor and anti-inflammatory properties have been attributed to the presence of key structural features such as an α,β-unsaturated ketone in ring A, a 5β,6β-epoxide in ring B, and a lactone side chain [1, 4, 7, 13, 30, 58–62]. Cysteine residues in the proteins are often implicated to react with these key electrophilic sites on the withanolide molecule [59, 60, 63, 64]. While other withanolides may possess α,β-unsaturated ketone and/or epoxide in some respect (e.g., paraminabeolides, capsisteroids, and chantriolides) and are bioactive, they are generally less potent than those withanolides possessing all three crucial functional groups.

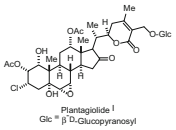
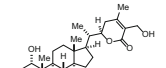
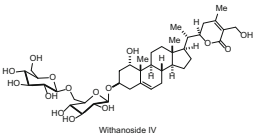
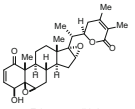
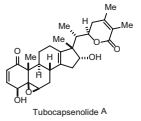
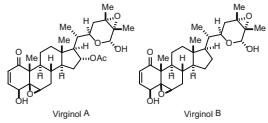
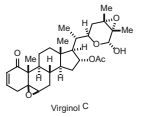
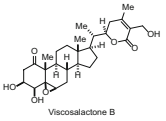
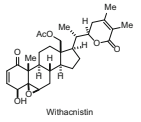
14.2 Modulation of Inflammatory Cell Signaling Pathways by Withanolides

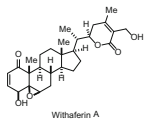
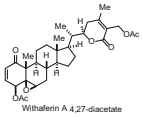
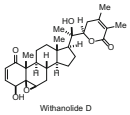
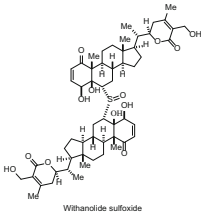
Inflammation is a complex immunological process by which our body fights against infection, cancer, or injury. The initial, acute stage of inflammation is mediated through the activation of immune cells, the resultant inflammatory cytokines and intracellular pathways. The initial immune mediators are $CD4^+$ T-cells or T-helper (Th) cells and are classified as Th-1, Th-2, and Th-17. They play a crucial role in regulating the cellular and humoral immune responses through recognition of antigens presented on antigen presenting cells via the major histocompatibility complex II. Th-1 cells promote the cellular immune response (macrophages) and primarily produce interferon (IFN)-γ, tumor necrosis factor (TNF)-β, and interleukin (IL)-2, whereas Th-2 cells promote the humoral immune system (antibodies) and primarily produce IL-4 and IL-10, and Th-17 cells help recruit neutrophils early in the adaptive response, produce IL-17 cytokine, and are involved in many autoimmune diseases [82]. The alteration of normal homeostasis of any of the Th cells through aberrant recognition of self or dysregulated production of cytokines plays a major role in the formation of chronic inflammatory or autoimmune/immunomodulatory diseases. Excess cytokine production leads to the over activation of multiple downstream inflammatory pathways, including Janus kinase

Table 14.1 Natural and semi-synthetic anti-inflammatory withanolides

Active compounds	Structures	Plant source	Targets inhibited/Inflammation models	Diseases involved	Ref.
Chantriolide A	 Chantriolide A Glc = β-D-Glucopyranosyl	*Tacca plantaginea* (Dioscoreaceae)*	TNFα-induced NF-κB	Inflammation	(29)
Cilistol G, Capsisteroids A and E	 Cilistol G  Capsisteroid A Capsisteroid E	*Solanum capsicoides* (Solanaceae)	superoxide anion and elastase release by human neutrophils	Inflammation and cancer	(9)
Coagulin L	 Coagulin L Glc = β-D-Glucopyranosyl	*Withania coagulans* (Solanaceae)	PPARγ, C/EBPα, and MCE	Obesity and metabolic syndrome	(65)
Daturafolisides A and B, Baimantuoluoside B, and 12-Deoxywithastramonolide	 Daturafolisides A Daturafolisides B  Baimantuoluoside B 12-Deoxywithastramonolide Glc = β-D-Glucopyranosyl	*Datura metel*(Solanaceae)	LPS-induced NO production in macrophages	Inflammation	(27)
Denosomin	 Denosomin	Synthetic	Increases vimentin, neuroprotective	Spinal cord injury	(66)
2,3-Dihydrowithaferin A and 4-(2,2-Dimethyl-3-oxocyclopropoxy)-2,3-dihydrowithaferin A	 2,3-Dihydrowithaferin A 4-(2,2-Dimethyl-3-oxocyclopropoxy)-2,3-dihydrowithaferin A	*Withania somnifera*(Solanaceae)	COX-2	Inflammation and cancer	(30)
6α,7α-Epoxy-1-oxo-5α,12α,17α-trihydroxywitha-2,24-dienolide	 6α,7α-Epoxy-1-oxo-5α,12α,17α-trihydroxywitha-2,24-dienolide	*Discopodium penninervium*(Solanaceae)	LTB_4 and COX-2	Inflammation and cancer	(26)
4β-Hydroxywithanolide E	 4β-Hydroxywithanolide E	*Physalis pruinosa* (Solanaceae)	NF-κB and MCP-1	Diabetes and obesity	(67)

3β-Hydroxy-2,3-dihydrowithanolide F		*Withania coagulans* (Solanaceae)	Formalin-induced arthritis model and cotton pellet granuloma method for sub-acute inflammation	Inflammation	(68)
Minabeolides-1, -2, -4, and -5		*Paraminabea acronocephala*(Alcyoniidae)	LPS-induced iNOS and COX-2 proteins in macrophages	Inflammation and cancer	(10)
Paraminabeolides A–D		*Paraminabea acronocephala*(Alcyoniidae)	LPS-induced iNOS protein in macrophages	Inflammation and cancer	(10)
Physalins A and O, and Isophysalin A		*Physalis alkekengi* (Solanaceae)	IKKβ, NF-κB, and LPS-induced NO production in macrophages	Inflammation	(64)
Physalins C, B, A, N, F and Withanolides E, F, G		*Physalis alkekengi and Physalis peruviana* (Solanaceae)	TNFα-induced NF-κB	Inflammation and cancer	(59)

Plantagiolide I	 Plantagiolide I Glc = β-D-Glucopyranosyl	*Tacca plantaginea* (Dioscoreaceae)*	PPARs	Inflammation and metabolic disorders such as diabetes and obesity	(29)
Sominone and Withanoside IV	 Sominone  Withanoside IV	*Withania somnifera*(Solanaceae)	Induces neurite outgrowth	Dementia and Alzheimer's disease	(39, 40)
Tubocapsanolide A	 TubocapsanolideA	*Tubocapsicum anomalum* (Solanaceae)	CCR7 expression, IKK, and NF-κB	Cancer	(69)
Tubocapsenolide A	 Tubocapsenolide A	*Tubocapsicum anomalum* (Solanaceae)	Hsp90-Hsp70 chaperone complex	Cancer	(62, 70)
Virginols A–C	 Virginol A Virginol B  Virginol C	*Physalis virginiana*(Solanaceae)	TPA-induced ear edema assay	Inflammation	(28)
Viscosalactone B	 Viscosalactone B	*Withania somnifera*(Solanaceae)	TNFα-induced NF-κB	Inflammation and cancer	(54)
			COX-2	Inflammation and cancer	(30)
Withacnistin	 Withacnistin	–†	STAT3 and STAT5	Inflammation and cancer	(71)
			TNFα-induced NF-κB and Ubiquitin proteasome pathway NF-κB and	Angio-inflammatory diseases and cancer	(55)

Withaferin A	 Withaferin A	*Withania somnifera*(Solanaceae)	zymosan-induced inflammed paw model	Inflammation and cancer	(24)
			NF-κB, AP-1, Nrf2, and IL-6	Inflammation and cancer	(72)
			COX-2	Inflammation and cancer	(30)
			STAT3	Inflammation and cancer	(53, 57)
			COX-2, PGE_2, STAT1, and STAT3	Microglial activation	(73)
			LPS-mediated HMGB1 release, ICAMs, and NF-κB	Vascular inflammatory diseases such as atherosclerosis	(51)
			NF-κB and AP-1	Inflammation and cancer	(56)
Withaferin A 4,27-diacetate	 Withaferin A 4,27-diacetate	Semisynthetic	TNFα-induced IKK and NF-κB	Inflammation and cancer	(54)
Withanolide D	 Withanolide D	*Withania somnifera*(Solanaceae)	Ubiquitin proteasome pathway and NF-κB network	Age-related macular degeneration	(52)
Withanolide sulfoxide	 Withanolide sulfoxide	*Withania somnifera* (Solanaceae)	COX-2 and TNFα-induced NF-κB	Inflammation and cancer	(74)

* Reference 28 had the genus Tacca assigned to family Taccaceae, which has been found in older texts but the APG II system has incorporated this genus into the family Dioscoreaceae. † Obtained from National Cancer Institute Developmental Therapeutics Program

(JAK)/STAT, NF-κB, phosphatidylinositol-3-kinase (PI3K)/Akt, and mitogen-activated protein kinase (MAPK). A number of withanolides have demonstrated significant immunomodulatory effects, including WS extract (primarily aqueous extract), withanolide A, physalins and coagulins; however, both immunostimulatory and inhibitory actions have been attributed to different withanolides. WS extract (primarily withanolide A and 2,3 dihydro-3-sulphonile withanone components) is able to shift the immune response toward Th-1 polarization, activate cytotoxic natural killer cells [83, 84], and recover depleted T-cells and increase expression of Th-1 cytokines IL-2 and IFN-γ in models of stress [32, 34, 85, 86]. Conversely, coagulins isolated from *Withania coagulans* and primarily coagulin-H have demonstrated immunosuppressive effects similar to prednisolone, inhibiting stimulated T and B-cell lymphocyte proliferation, and Th-1 cytokine production

Table 14.2 Anti-inflammatory activity of withanolides in plant extracts

Plant extract	Plant part	Active components	Effects	Therapeutic uses	References
Physalis peruviana (Solanaceae)	Fruits	Withanolides, polyphenols, and phytosterols	Antioxidant, anti-inflammatory, and renoprotective	Acute renal injury	[75]
	Calyces	–	Anti-inflammatory and immunosuppressive effect on macrophages apoptopic (downregulates IL-6, TNF, and MCP-1)	Inflammation	[76]
Withania somnifera (Solanaceae)	Roots	Withanolides such as withanolide A and alkaloids	Neuroprotective	Alzheimer's disease	[77]
	Roots	Withanolides, alkaloids, and flavanoids	Immunomodulatory, anti-inflammatory, and proapoptopic (downregulates IL-6, IL-1β, IL-8, Hsp70, and STAT-2)	Cancer	[78]
	Roots	–	Antioxidant, anti-inflammatory, and cytoprotective	Inflammatory bowel disease	[79]
	Leaves	–	Neuroprotective against glutamate neurotoxicity	Stroke and neurodegenerative disorders	[80]
Withania coagulans (Solanaceae)	Fruits	–	Antioxidant, anti-inflammatory, antihyperglycemic, immunomodulatory, and renoprotective (downregulates IL-1β, IL-6, TNFα, IL-4, and IFN-γ)	Diabetes and associated renal complications	[81]

possibly through IL-2 receptor binding [87, 88]. Similar to coagulins, physalins B, F, G, and H isolated from *Physalis angulata* have demonstrated immunosuppressive properties. Physalins B, F, and G showed inhibition of lipopolysaccharide (LPS)-activated macrophages along with their cytokine (TNF-α, IL-6 and IL-12) and nitric oxide (NO) production, whereas concanavalin A-induced T-cell proliferation and cytokine production in a mechanism distinct from dexamethasone [89, 90]. Physalin H has demonstrated polarization to Th-2 cells with inhibition of Th-1 cytokines IL-2 and IFN-γ, increased Th-2 cytokines IL-4 and IL-10, and induction of the heme oxygenase-1 [91].

There are strong preclinical and clinical studies that demonstrate that inflammation initially started by immune cell mediators may persist chronically, resulting in ongoing stimulation of inflammatory mediators and regulatory pathways that contribute to the pathogenesis of chronic diseases including cardiovascular, neurologic, and pulmonary diseases, as well as cancer, diabetes, and obesity [92–94]. As with acute inflammation, chronic inflammation is mediated through various signaling factors, which include proinflammatory cytokines such as TNFα, IL-1, IL-6, IL-8, IL-12, NO, adhesion molecules, and chemokines [95]. Additionally, transcription factors that regulate the expression of inflammatory mediators such as NF-κB, activator protein 1 (AP-1), peroxisome proliferator-activated receptor (PPAR)-γ, STAT3, hypoxia inducible factor-1 (HIF-1), β-catenin/Wnt, hedgehog, and nuclear factor erythroid 2-related factor 2 (Nrf2) have been linked to chronic diseases. The DNA-binding capacity of these transcription factors is modified by several signaling cascades such as JAK/STAT, MAPKs, PI3K/Akt/mechanistic target of rapamycin (mTOR), and ubiquitin proteasome system [96]. These signaling pathways have a wide range of functions and show complex crosstalk depending on the cell type and the chronic disease involved. Withanolides have emerged recently as potential therapeutics for chronic diseases due to their unique ability to modulate multiple signaling pathways. In this chapter, we will discuss several different withanolides and their seemingly broad mechanism of action in modulating key molecular pathways that connect inflammation and chronic diseases.

14.2.1 Withanolides Modulate the NF-κB Pathway

The NF-κB family of transcription factors plays a prominent role in the immune system and inflammation. In response to ligation by Toll-like receptors (TLRs) and IL-1 receptor family members (B-cell and T-cell receptors), NF-κB regulates the expression of several factors such as inhibitor of apoptosis protein-1 (IAP1), inducible nitric oxide synthase (iNOS), cytokines, cyclooxygenase (COX)-2, prostaglandins, growth factors, and effector enzymes [97–99]. NF-κB is activated by inflammatory cytokines, stress, free radicals, radiation, growth receptors, and TNFα leading to transcriptional regulation of several genes that are involved in proliferation, inflammation, cellular survival, apoptosis, angiogenesis, and differentiation [100–103]. The mammalian transcription NF-κB family of proteins includes RelA (p65), RelB, NF-κB2 (p52), c-Rel, and NF-κB1 (p50). In the absence of stimuli, inactive forms of NF-κB proteins are present in the cytoplasm due to their interaction with several inhibitors of kappaB (IκB) proteins. Upon exposure to stimuli, NF-κB is activated either through canonical or noncanonical pathways by regulatory IκB kinases (IKK) such as IKKα, IKKβ, and IKKγ. In the most common classical pathway, IKK phosphorylation leads to IκBα phosphorylation at serine 32 and serine 36, followed by phosphorylation, nuclear localization, DNA binding of p65–p50 complex, and transcriptional activation of NF-κB responsive genes [104].

Activation of NF-κB is essential for the survival and expression of inflammatory mediators. Hence, constitutively active NF-κB is associated with several inflammation-mediated chronic diseases such as cancer, neurological, and metabolic disorders [98].

A number of naturally occuring withanolides such as chantriolide A, physalins A, B, C, and O, viscosalactone B, WA, withanolides D, E, F and withaferin A 4,27-diacetate, a diacetyl derivative of WA have been reported to modulate the regulation of NF-κB [24, 29, 34, 52, 54, 59, 64]. Several studies have examined the beneficial effect of inhibiting transcriptional activity of NF-κB in chronic inflammatory diseases including cancer [24, 55, 56, 72]. In a study led by Aggarwal et al., various withanolides isolated from the leaf extract of WS along with their semi-synthetic acetylated derivatives were tested for their inhibitory effects on NF-κB activation induced by activators such as cigarette smoke condensate, TNF, doxorubicin, and IL-1β [54]. Withaferin A and viscosalactone B along with their 4,27-diacetyl derivative inhibited TNF-induced NF-κB activation in human myeloid leukemia KBM-5 cells, whereas physagulin D and related glycosidic withanolides were inactive. The mechanism through which the above withanolides blocked NF-κB was through the inhibitory effects of activated IκBα, with subsequent phosphorylation of IκBα and p65, followed by prevention of IκBα degradation. Blocking the degradation of IκBα in turn prevents nuclear localization of p65, and activation of NF-κB-dependent gene products such as Bcl-2-related protein A1 (Bfl-1/A1), IAP-1, c-FADD-like cFLIP, ICAM1, and COX-2 [54]. To further understand the SAR, the authors examined TNF-induced NF-κB activity after treatment of human myeloid leukemia KBM-5 cells with various withanolides isolated from the leaf extract of WS along with their synthetically modified acetylated derivatives by electrophoretic mobility shift assay (EMSA). This analysis pointed toward the importance of the α,β-unsaturated ketone in ring A as a required structure for the potent inhibition of NF-κB activity [54].

Physalins A, L, G and O, and isophysalin A isolated from *Physalis alkekengi* were evaluated for their anti-inflammatory properties and Physalins A, O and isophysalin A showed significant inhibition of LPS-induced NO production in macrophages [64]. Based on their structural features characterized by the presence of either an α,β-unsaturated ketone in ring A (physalin O) or an α,β-unsaturated ester in lactone side chain featuring an exomethylene group (physalin A and isophysalin A), these physalins were able to conjugate with glutathione as identified by ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) analysis. Furthermore, peptide mapping and sequencing of alkylated IKKβ using micrOTOF-MS revealed alkylation of six cysteine residues on IKKβ by physalin A indicating IKKβ as a potential target for its anti-inflammatory mechanism of action. In another related and complementary study, SARs were performed on a library of withanolides including the physalins. This study revealed the importance of the oxygenated right-side partial structure (including the lactone side chain) and the 5β,6β-epoxide, or C5–C6 olefin in the B-ring for the inhibition of NF-κB activation [59]. Withanolides and physalins with 5β,6β-epoxide inhibited NF-κB signaling through prevention of IκBα degradation and p65 nuclear

localization, whereas those with C5–C6 olefin inhibited NF-κB function by blocking p65/p50 dimer binding to DNA [59].

Chantriolide A, one of the eight compounds isolated from *Taccaplantaginea* exhibited potent inhibition of TNFα-induced NF-κB transcriptional activity in human hepatocellular carcinoma (HepG2) cells in an NF-κB-luciferase assay [29]. In another study, withanolide D and WA from WS inhibited angiogenesis through blocking of NF-κB activity by suppressing proteasome-mediated ubiquitin degradation of IκBα in human umbilical vein endothelial cells [52]. Additionally, 4β-hydroxywithanolide E was shown to inhibit inflammatory response in adipocytes via inhibition of NF-κB transcriptional activity [67]. Inhibition of IKKβ activation by 4β-hydroxy withanolide E through suppression of IKKβ phosphorylation was mechanistically distinct from the NF-κB inhibition observed for WA, where the induction of IKKβ over-phosphorylation was shown to inhibit IKKβ activation [24]. Moreover in vivo, 4β-hydroxy withanolide E demonstrated an improvement of impaired glucose tolerance suggesting its potential role for the treatment/prevention of metabolic disorders including type 2 diabetes [67]. Overexpression of CCR7 in metastatic breast cancer cells has been associated with lymph node metastasis [69]. In breast cancer cells MDA-MB-231, tubocapsanolide A inhibited TAK1 to suppress NF-κB-mediated CCR7 expression leading to the inhibition of lymphatic invasion of breast cancer in vitro and in vivo.

In addition to the inhibition of NF-κB activation, WA and several other withanolides have been shown to directly block the expression of LPS- or TNFα-induced NF-κB-regulated inflammatory genes such as iNOS, COX-1, COX-2, and NO [10, 13, 14, 25, 26, 64, 74]. Nitric oxide is a small molecule that regulates MMPs and joints extracellular matrix, and is modulated through iNOS. COX-1 and COX-2 convert arachidonic acid to prostaglandins, which in turn cause a significant inflammatory response. COX-1 is constitutively expressed in most cell types, and is responsible for maintenance of normal physiologic function, whereas COX-2 is inducible in response to proinflammatory cytokines [26]. Nair and co-workers were the first to demonstrate the role of withanolides in inhibiting COX enzymes and provide insight into their anti-inflammatory mechanism [30]. Withaferin A, viscosalactone B, 2,3-dihydrowithaferin A, and 4-(2,2-dimethyl-3-oxocyclopropoxy)-2,3-dihydrowithaferin A were shown to inhibit COX-2 enzyme but not COX-1. Interestingly, during this study it was observed that the presence of a double bond between C-24 and C-25 in the lactone ring was essential for COX inhibitory activities and a withanolide lacking this unsaturation in the lactone ring was found to be inactive against both COX-1 and COX-2 enzymes [30].6α,7α-Epoxy-1-oxo-5α,12α,17α-trihydroxywitha-2,24-dienolide from *Discopodiumpenninervium* was found to inhibit COX-2 and leukotriene B_4 (LTB_4) but was inactive against the COX-1 enzyme [26]. Like other withanolides, withanolide sulfoxide, a sulfoxide dimer of WA was highly selective in inhibiting COX-2 compared to COX-1[74].

Daturafolisides A and B along with other known withanolides from *Datura metel* were shown to exhibit significant reduction in NO production in LPS-induced RAW 264.7 macrophage cells [27]. Of note, both of these compounds lack the

α,β-unsaturated ketone in ring A and the 5β,6β-epoxide in ring B, however, they do possess a δ-lactone side chain. Additionally, withanolides such as paraminabeolides and minabeolides obtained from a marine source were found to inhibit LPS-induced iNOS expression in RAW 264.7 macrophages, with minabeolides also effectively inhibiting COX-2 expression [10].

14.2.2 Withanolide Modulation of the JAK/STAT Pathway

The JAK/STAT pathway is a key-signaling mediator of cytokines and growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), IL6, as well as oncogenic proteins [105]. Activation of STAT proteins depends on their binding to cytokines and growth factor receptors on the plasma membrane followed by tyrosine phosphorylation either directly by receptor tyrosine kinases (RTKs) or by non RTKs such as JAK or Src [106, 107]. Upon phosphorylation, cytoplasmic STAT proteins undergo dimerization via reciprocal SH2-domain/phosphotyrosine interactions followed by translocation to the nucleus for DNA binding to STAT-specific response elements leading to transcriptional activation. There are eight known STAT proteins (STATs 1A, 1B, 2, 3, 4, 5A, 5B, and 6) that play diverse biochemical roles in several important processes such as survival, proliferation, apoptosis, invasion, immune response, inflammation, and angiogenesis [105, 108, 109]. Among the eight isoforms STAT3 and STAT5 are constitutively activated in several solid tumors, including lung, bladder, breast, colon, as well as in hematological malignancies [108]. Additionally, STAT3 is also interconnected with the NF-κB pathway and plays a central role in inflammation [107].

Several chronic diseases including cancer have been shown to induce aberrant regulation of STAT3. This transcription factor promotes oncogenic processes such as invasion, metastasis, and angiogenesis as several genes involved in these mechanisms such as cyclin D1, c-Myc, vascular endothelial growth factor (VEGF), mucin 1 (MUC-1), twist family BHLH transcription factor (TWIST) are all regulated by STAT3 [110–114]. Studies have investigated the role of WA from WS in regulating STAT proteins in different cancer models including colon cancer, breast cancer, multiple myeloma, and neuroblastoma [53, 57, 115, 116]. In breast cancer, WA treatment of triple negative MDA-MB-231 and hormonally active MCF-7 cells effectively decreased the constitutive as well as the IL-6 inducible phosphorylation of JAK 2 and its downstream target STAT3 thereby inhibiting the transcriptional activity of STAT3 [115]. In renal carcinoma Caki cells, WA had a similar effect and also downregulated the expression levels of anti-apoptotic proteins that are regulated by STAT3 like Bcl-2, cyclin D1, survivin, and Bcl-xL, thereby inducing apoptosis [116]. Docking studies showed that WA not only downregulates the phosphorylation of STAT3 at the tyrosine Y705, but also prevents dimerization of STAT3 [57]. In addition to cancer cells, WA also is able to suppress the phosphorylation of STAT1/3 in murine BV2 microglial cells, leading to a reduction

in LPS-induced COX-2 downregulation and PGE_2 production [73]. Withacnistin, an unmodified withanolide blocked both IL-6 as well as EGF-stimulated binding of STAT3 and STAT5 to gp130 and EGF-receptor (EGFR) in MDA-MB-468 breast cancer cells. This resulted in subsequent downregulation of STAT3 tyrosine phosphorylation and decreased nuclear translocation. Further evaluation of STAT3-DNA binding and transcriptional activity after Withacnistin treatment revealed blocking of both DNA binding as well as STAT3 reporter activity. This in turn caused downregulation of STAT3 target genes Bcl-xL and MCL-1 resulting in apoptosis [71].

14.2.3 Modulation of the AP-1 Pathway by Withanolides

The transcription factor AP-1, which plays a key role in the inflammatory response is implicated in several diseases such as cancer, psoriasis, inflammatory bowel disease (IBD), rheumatoid arthritis (RA) and fibrosis [117]. The AP-1 complex consists of homo and hetero dimers of Jun (JunD, C-Jun, and JunB) and the Fos (FosB, C-Fos, Fra-1 and Fra-2) family of proteins [118, 119]. Cytokines, chemokines, hormones, and growth factors as well as external stress factors are known to activate AP-1 signaling. The AP-1 complex translocates to the nucleus in response to stress signaling cascades, such MAPKs and c-Jun terminal kinases [120]. This in turn leads to activation of AP-1 and regulates multiple functions such as differentiation, transformation, proliferation, and survival [121]. The crude ethanol extract of WS has been shown to inhibit the nuclear localization of both AP-1 and NF-κB in LPS-activated peripheral blood mononuclear cells (PBMC) of both normal and RA patients, as well as synovial fluid mononuclear cells (SFMC) of RA patients. This in turn led to decreased downstream transcription target genes such as MMPs, COX-2, and iNOS, all of which are known mediators of RA [122].

14.2.4 Withanolides Can Modulate the PPARγ Pathway

PPARγ was first discovered in adipocyte differentiation and lipid metabolism and is one of three members in this nuclear receptor family of transcription factors [123]. The other members of the PPARs in mammals are PPARα and PPARβ/δ. The PPARs activate several genes involved in inflammation, adipogenesis, lipid metabolism, glucose metabolism, cellular differentiation, development, and tumorigenesis via binding of the PPAR/retinoid X receptor heterodimer to PPAR-responsive regulatory elements [124, 125]. PPARγ plays a key role in inflammation through modulation of proinflammatory transcription factors such as NF-κB and AP-1 [113]. Treatment of 3T3-L1 adipocytes with WA resulted in phosphorylation of extracellular signal-regulated kinase (ERK), followed by decreased expressions of PPARγ leading to altered levels of Bcl_2 and Bax expression, induction of apoptosis,

and inhibition of adipogenesis [126]. In addition to WA, other withanolides such as plantagiolide J and I isolated from *Taccaplantaginea* [29] and coagulin-L isolated from *Withania coagulans* [65] also modulate PPARγ transcriptional activity.

14.2.5 Modulation of the Hsp90 Pathway by Withanolides

Heat shock proteins (Hsp) are ATP-dependent ubiquitously expressed molecular chaperones that are involved in the folding, assembly, maintenance, and transport of key regulatory proteins involved in numerous signaling pathways in the cell. Several environmental and physiological stimuli such as hypoxia, oxidative damage, inflammation, infection, and elevated temperature induce the expression of these highly conserved molecular chaperone family of proteins as a protein homeostasis and survival response [70, 114]. The Hsp90 family of proteins (Hsp90α, Hsp90β, GRP94, and TRAP1) form a large complex with other co-chaperones such as cdc37, HSP70-HSP90 organizing protein, p27, Hsp32, and Hsp70. This complex then stabilizes and maintains functional activity of proteins/kinases in many key signaling pathways, such as PI3K/Akt/mTOR, p38/MAPK, and NF-κB, all of which play critical roles in inflammation, chronic inflammatory diseases, and oncogenesis. Through inhibition of Hsp90, and therefore inhibition of its oncogenic chaperone clients, cancer cells undergo apoptosis [124, 125].

Several studies have shown that withanolides such as WA, withalongolides A and B, tubocapsenolide A, and some of their synthetically modified analoges such as withalongolide A triacetate and withalongolide B diacetateare are able to target multiple cancers such as colon, prostate, brain, breast, head and neck, skin, adrenal, and thyroid both in vitro and in vivo [17, 20, 21, 53, 63, 70, 127–137]. Withanolides such as WA and withalongolide A are known to block Hsp90 chaperone function through blocking the Hsp90/cdc37 complex, and induction of thiol-mediated oxidative stress [63, 138, 139]. The Hsp90/cdc37 complex facilitates active conformation of client kinases in particular, such as Akt, cyclin-D1, raf-1, and cdk4. Blocking this complex leads to dysfunctional or proteasome mediated degradation of these kinases within multiple oncogenic, pro-survival, and proliferative kinase cascades (p38/MAPK, PI3K/Akt/mToR, NF-κB pathways), which ultimately leads to cancer cell apoptosis [136, 138]. In addition to targeting the bulk cancer cell population, WA and withalongolide A triacetate may also target the cancer stem cell (CSC) population. These CSCs comprise a small fraction of cancer cells, and are characterized by their tumor initiating and self-renewal capacity. WA and withalongolide A triacetate block several developmental pathways such as Wnt/β-catenin, notch, and NF-κB, as well as vimentin and VEGF, all of which are important in inflammation, self-renewal, and CSC epithelial-to-mesenchymal transition [115, 140–145].

14.2.6 Withanolide Modulation of Nrf2 Pathway

Nuclear factor erythroid 2 related factor 2(Nrf2) is a transcription factor that regulates genes involved in redox homeostasis, inflammation, energy metabolism and cellular growth [146]. Under normal homeostatic conditions, Nrf2 is anchored in the cytoplasm as a complex with Kelch like ECH-associated protein-1 (Keap1), which facilitates ubiquitin mediated proteasome degradation of Nrf2 and decreased expression of Nrf2 target genes. However, in response to stimulation by growth factors, electrophilic stressors, and changes in redox signal, Nrf2 ubiquitination is disrupted and levels increase rapidly. Nrf2 translocates to the nucleus and upregulates expression of proteins involved in glutathione and thioredoxin-based antioxidant defense, drug metabolism and efflux, and proteins associated with heme and iron metabolism [147]. Nrf2 is engaged in crosstalk with several signaling pathways that play a critical role in the pathogenesis and progression of chronic diseases, including NF-κB, PI3K, MAPK, glycogen synthase kinase-3β, and notch [146, 147]. Molecular docking studies have shown that both WA and withanone interact with the amino acids Ala 69, Gln 75, and Phe 71 of Nrf2 [148]. In another study, WA induced reactive oxygen species (ROS) that activated JNK and stabilized Nrf2 that resulted in activation of NADPH quinone oxidoreductase and Tap73 transcriptional function leading to apoptosis of cancer cells [149]. WA was also shown to inhibit NFκB, AP-1, and Nrf2 in adriamycin-resistant human myelogenous erythroleukemic K562/Adr cells in a dose-dependent manner [72]. Moreover, compared to other tested natural products such as quercetin, only WA overcomes attenuated caspase activation and blocking of apoptosis in K562/Adr cells [72].

14.2.7 Modulation of the HIF-1 Pathway by Withanolides

Under normal oxygen conditions, the HIF-1 α protein is synthesized at a high rate and rendered transcriptionally inactive due to immediate hydroxylation-dependent proteasome/ubiquitin degradation by the VHL E3 ligase. However, when hypoxia is induced through impaired cellular oxygen balance, hydroxylase activity is downregulated, HIF-α protein is stabilized, and HIF-1 is activated [150]. Transcriptional activation of HIF-1 upregulates several genes that control glycolytic metabolism, angiogenesis, invasion, metastasis, and cell survival, such as VEGF, MMPs, stromal cell-derived factor-1, e-cadherin, chemokine receptor 4, EGF, and transforming growth factor beta (TGF-β) 3 [151–155]. Crosstalk between NF-κB and HIF pathways has been shown to be associated with several chronic inflammatory diseases such as cancer, RA, asthma, and chronic obstructive pulmonary diseases [156]. In solid tumors, the availability of oxygen within the tumor decreases as distance from blood vessels increases resulting in the creation of hypoxic regions [157]. This is known to be responsible in part for therapy resistance

and metastatic spread [158]. Although, no study thus far directly demonstrates inhibition of HIF-1 transcriptional activation by withanolides, a few note down-regulation of migration-promoting HIF-mediated genes such as VEGF, heterogeneous nuclear ribonucleoprotein K (hnRNP-K) and MMPs, which lead to restriction of angiogenesis and metastasis [159].

14.3 Withanolides for Clinical Development

As discussed above, studies show the ability of withanolides to target multiple interconnected signaling pathways such as PI3K/Akt/mTOR, JAK/STAT, AP-1, NF-κB, PPARγ, Nrf2 and MAPK. Withanolides target these pathways through multiple mechanism, such as blocking Hsp90-Cdc37 co-chaperone interaction, targeting Akt and its downstream pathways, and induction of thiol-mediated oxidative stress (summarized in Fig. 14.3). Each of these mechanisms and pathway interactions play important roles in the development of chronic inflammatory diseases. Building on the studies identifying mechanisms of action of withanolides, we will discuss the clinical importance of withanolides on inflammatory mediated diseases including chronic inflammatory/autoimmune, cancer, and neurologic.

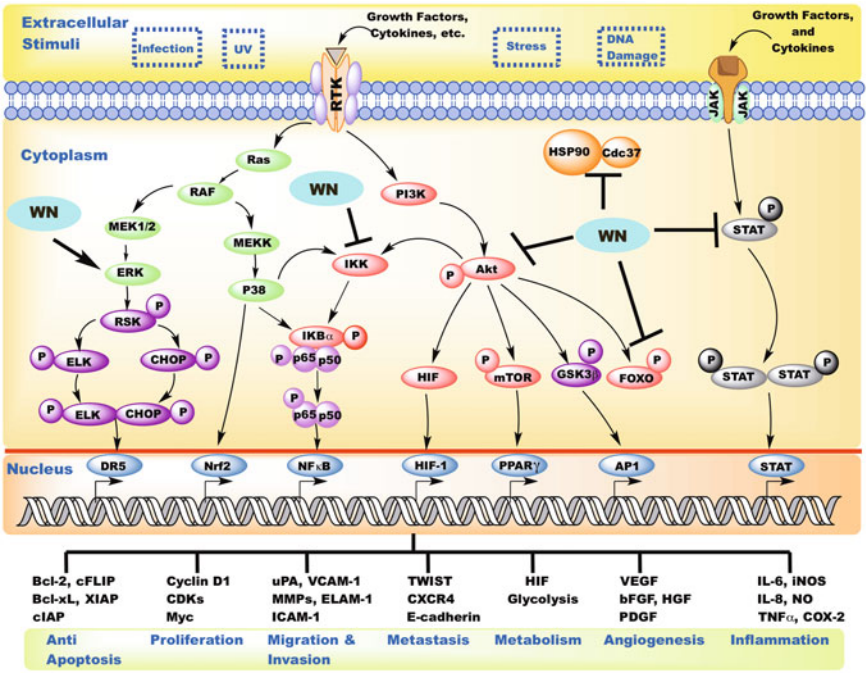


Fig. 14.3 Schematic diagram representing modulation of various inflammatory pathways by withanolides

14.4 Application of Withanolides in Inflammatory/Autoimmune Diseases

14.4.1 Osteoarthritis and Rheumatoid Arthritis

In Ayurvedic medicine, withanolides are frequently used to treat both osteoarthritis (OA) and RA and there are several anti-inflammatory pathways affected by withanolides that contribute to chondro protection and treatment. The previously described NF-κB pathway plays a key role in arthritic inflammation, as do the downstream effectors NO via iNOS, COX-1, and COX-2 enzymes [160]. NO has been implicated in chondrocyte apoptosis in RA [161] and WS extracts have demonstrated reductions in NO in murine macrophage cell lines [122] and human chondrocytes [162]. Nonsteroidal anti-inflammatory drugs nondiscriminately inhibit COX enzymes, which with chronic use increases the risk of upper GI ulcers. Withanolides have been demonstrated to exhibit significant selective COX-2 inhibition while sparing COX-1 [13, 26, 163]. Additionally, along a parallel proinflammatory pathway with arachidonic acid, withanolides inhibited the production of LTB_4 [26].

Another important mediator of arthritis is the formation of ROS leading to oxidative stress, and the protective role of Nrf2 pathway in glutathione and thioredoxin-based antioxidant defense [164]. However, the exact mechanism by which withanolides act within this pathway is still unclear as studies have demonstrated that withanolides are both ROS protective through inhibition of lipid peroxidation [13, 163] and can induce oxidative stress in rabbit chondrocytes [165, 166].

14.4.2 Therapeutic Benefits of Withanolides for Osteoarthritis and Rheumatoid Arthritis In Vivo

There have been several studies indicating that withanolides have a chrondroprotective aspect. WS extract has been shown in animal models to significantly reduce the effects of collagenase on bovine Achilles tendons with even a suggestion of collagen stabilization [167]. This has also been demonstrated with inhibition of the gelatinase activity of collagenase type 2 enzyme in vitro [168]. Several collagen-induced arthritic rat models have noted significant amelioration of paw and ankle arthritis, oxidative stress, degradation of cartilage, and improvements in functional recovery and radiological score [24, 169–171]. Aqueous extracts of WS have demonstrated a significant dose-dependent reduction in adjuvant-induced hind foot pad thickness, reductions in immune complement activity [172], as well as reduced arthritic index, autoantibodies, and CRP levels with results comparable to methotrexate treatment. These rats similarly demonstrate reductions in oxidative stress through decreased lipid peroxidation, glutathione-S-transferase activity, and

an increase in glutathione content and ferric-reducing ability [170]. In a monosodium urate crystal-induced rat model of gout, WS extract demonstrated a significant reduction in paw pad volume down to that of normal controls as well as analgesic and antipyretic effects without any evidence of gastric injury [173]. Despite the conflicting reports of WA increasing oxidative stress and reducing type II collagen through induction of COX-2 by microRNA-25 [166], the majority of evidence continues to support the chondroprotective and analgesic properties of withanolides in arthritis.

14.4.3 Clinical Activity of Withanolides for Osteoarthritis and Rheumatoid Arthritis

On the basis of the long-standing use of WS extract in the treatment of arthritis in Ayurvedic medicine and the support from animal models, there have been several human tissue and blood studies performed in recent years. WS root powder extracts were given to patients with chronic OA and mild to moderate (Grade 1–2) articular cartilage and a portion of the cartilage was explanted for analysis. Half of the cartilage samples had a significant short-term chondroprotective response as demonstrated by significant decreases in proteoglycan release [168]. Further research on the explanted cartilage also demonstrated a significant reduction in NO production as an inflammatory regulator molecule in 50 % of the patient samples [162].

In RA, WS crude ethanol extract significantly inhibited LPS-induced expression of multiple proinflammatory cytokines from PBMCs and SFMCs taken from RA and normal patients, including TNF-α, IL-1β, and IL-12 p40. The reduction in inflammatory cytokines may have resulted from suppression of LPS-activated NF-κB and AP-1, in addition to inhibition of AP-1 nuclear translocation and LPS-induced phosphorylation of IκBα [122].

Although there have been several human trials for OA and RA, they were developed using combinations of Ayurvedic drugs. Combination therapy RA-11 (that includes WS, *Boswelliaserrata*, *Zingiberofficinale*, and *Curcumalonga*) was given to patients in a randomized, placebo-controlled OA trial. Results demonstrated a mean reduction in pain (using visual analog scale) and in the modified WOMAC index (Western Ontario McMaster University OA Index) at 16 and 32 weeks compared to placebo without any significant in adverse events [174]. A pilot prospective study of the combination Ashwagandha and SidhMakardhwaj in RA demonstrated a significant ACR20 (20 % improvement in tender joint/swollen join counts and 20 % reduction in 3 of 5 areas, physician global assessment score, patient global assessment score, pain assessment score, patient self-assessed disability index score, and ESR) response in 56 % of patients, and a moderate response in 40 % of patients by EULAR criteria (European League Against Rheumatism) [175]. Although only part of a combination therapy and the effects of WS alone cannot be determined, these trials suggest that withanolides

may have chondroprotective, anti-inflammatory, and analgesic characteristics in human joints without significant adverse events.

14.4.4 Applications in Systemic Lupus Erythematous and Inflammatory Bowel Disease

The pathogenesis of systemic lupus erythematous (SLE) and IBD is rooted in chronic, aberrant activation of the immune system and inflammatory pathways. SLE is a complex B-cell mediated autoimmune disease characterized by the generation of autoantibodies against nuclear antigens (antinuclear antibody) and a type III hypersensitivity reaction (antibody–antigen complexes) leading to chronic inflammation and deposition of the antibody–antigen complexes within small vessels of end organs, such as the kidneys, skin, and brain [176]. In IBD, although chronic, intermittent inflammation is a cornerstone of disease progression, the pathogenesis is more complex, involving genetic susceptibilities, perturbation of mucosal physiology/epithelial barrier, and homeostasis of intestinal innate immune system [177]. For both SLE and IBD, current therapies are based upon anti-inflammatory, immunosuppressive, or immune/biologic therapies. Ongoing therapeutic research is focused on identifying additional immune targets including modulation of the Th-1, Th-2 and Th-17 responses, inflammatory cytokines, and downstream inflammatory pathways [178, 179]. As discussed previously, withanolides including *Withania coagulin,* and *Physalis angulata* have demonstrated significant immunomodulatory effects on Th cells and inflammatory cytokines making these compounds an exciting new area of therapeutic development for inflammatory and autoimmune diseases.

14.4.5 Therapeutic Benefits of Withanolides for SLE and IBD In Vivo

The recent characterization of the effects of withanolides on immunomodulation has given rise to several in vivo animal models of SLE and IBD. Aqueous WS extracts were used to treat a nonautoimmune pristane-induced model of SLE in mice, which develop SLE-like symptoms, including autoantibody production, proteinuria, and nephritis. The prophylactic effects of WS extract were characterized by orally administering WS for one month prior and six months following pristane injections. WS treatment resulted in significant reductions in ROS formation within intraperitoneal macrophages, as well as reductions in inflammatory cytokines (IL-6 and TNF-α), and decreased inflammation within the kidneys, liver, and lung, however, no reduction in the humoral response autoantibodies or immune complex deposition [180, 181].

Using trinitrobenzylsulfonic acid (TNBS)-induced IBD model in rats, a rectal gel formulation from aqueous WS extract was administered from the fourth to fourteenth day, and demonstrated a dose-dependent inhibition of lipid peroxidation up to 96 %, hydrogen peroxide scavenging up to 82 %, and NO scavenging ability similar to curcumin. Histopathology demonstrated a significant decrease in colonic injury with WS treatment along with improvement in macroscopic damage when compared to untreated controls. WS-treated rats also retained their weight with improved recovery following induction of the TNBS model [79].

While the animal data for these inflammatory and autoimmune diseases remains relatively sparse, there has yet to be any major human studies using withanolides in these diseases. In the meantime, the immunomodulatory effects of withanolides continue to be characterized and hold therapeutic potential for the treatment of a number of autoimmune and immune-modulating diseases.

14.5 Application of Withanolides in Cancer Therapy

The discovery that most withanolides have cytotoxic capabilities against a wide range of cancers has initiated a large breadth of research into the cytotoxic mechanisms and potential therapeutic benefits of withanolides for cancer treatment and prevention. As previously described and depicted in Fig. 14.3, withanolides inhibit multiple aspects of inflammatory pathways. While these inflammatory pathways have complex interactions between one another, they also interact with proliferative and oncogenic pathways. Several key mechanisms of cytotoxicity from withanolides have been described and these include: (a) induction of oxidative stress, (b) inhibition of proteasome mediated ubiquitin degradation of IκBα (leading to inhibition of NF-κB and its downstream effectors AP-1 and Nrf-2), (c) inhibition of transcription factor STAT3, (d) inhibition of Hsp90 (by blocking interaction of the Cdc37 co-chaperone with Hsp90) with resultant Hsp90 client inhibition in the PI3K/Akt and MAPK pathways, (e) dysregulation of cytoskeletal and structural proteins, and (f) angiogenesis inhibition though HIF-1 [1, 137, 182, 183]. Withanolides with significant activity and identified mechanisms are depicted in Table 14.1, and their cytotoxic characteristics have been demonstrated in multiple cancer types, including breast, ovarian, colon, head and neck, renal, prostate, pancreatic, thyroid, glioblastoma, and hematologic cancers such as lymphoma, leukemia, and multiple myeloma [1, 70, 136, 182–186].

14.5.1 Anticancer Benefits of Withanolides in Animal Models

The groundwork done in vitro to establish the cytotoxic effect of withanolides against cancer cell lines and characterize their multiple mechanisms of therapeutic

potential led to the translational evaluation of these compounds in several cancer animal models. Since cancer is a heterogeneous complex disease process, different cancers utilize different key oncogenic pathways for survival. Because different aspects of the inflammatory pathways are important in various cancer models, withanolides demonstrate cytotoxic effects on tumor growth, rate of metastatic spread, and even prevention. Looking at the STAT3 pathway in a colorectal cancer in vivo model, WS extract treatment attenuated IL-6 activation of STAT3 and demonstrated a 1.44 fold decrease in average tumor volume [53].

Following the initial characterization of a novel withanolide tubocapsenolide A to inhibit Hsp90–Hsp70 complex function in breast cancer through direct thiol oxidation [70], WA was shown to bind to the C-terminal of Hsp90, disrupt the Hsp90–Cdc37 complex, and inhibit chaperone activity in pancreatic cancer. Pancreatic cancer xenografts treated daily with withanolides demonstrated a 58 % reduction in average tumor volume compared to controls [63]. In medullary thyroid cancer, RET, a known Hsp90 client, is a key proto-oncogene that encodes for a transmembrane RTK. WA treatment of medullary thyroid xenografts not only inhibited RET phosphorylation and activation, but also inhibited phospho-Akt and phospho-ERK protein expression ex vivo. The xenograft tumors also demonstrated significant, 2-week delayed growth kinetics with 80 % of mice responding to WA treatment, as well as improved survival when compared to controls [187]. Withanolide E has also been shown to sensitize renal carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis through cFLIP degradation as a result of inhibited Hsp90 chaperone function. Combination treatment of mouse renal carcinoma xenografts with withanolide E and a TRAIL death receptor agonist demonstrated complete and sustained tumor responses in 55 % of treated mice compared to control or either treatment alone [188].

Cytoskeletal and structural protein inhibition by withanolides has been shown to lead to cell cycle arrest, decreased epithelial-to-mesenchymal transition (EMT), and decreased metastasis. In breast cancer, WA binds to and inhibits β-tubulin, decreasing β-tubulin protein levels in G_0–G_1 phase cells and severely disrupting normal spindle morphology, both of which lead to cell cycle arrest [14]. Withaferin A also induces perinuclear vimentin (a mesenchymal protein) accumulation leading to rapid vimentin depolymerization and disrupted morphology. In both murine mammary carcinoma and human xenograft breast cancer models in mice, WA and WS treatment showed a dose-dependent inhibition of tumor growth kinetics, a decrease in number of metastatic lung nodules, and an increase in ser56 phosphorylated vimentin that is indicative of disassembly [189, 190]. Subsequent studies also showed that WA treatment attenuated TNF-α and TGF-β induced EMT, increased the epithelial phenotype protein E-cadherin, and inhibited vimentin expression in addition to inhibited tumor grown kinetics and cell proliferation [23, 143]. Together, these studies characterize the ability of WA to inhibit EMT both in vitro and in vivo, which may inhibit the metastatic potential of these tumors.

Another area of research in cancer therapy is the inhibition of angiogenesis which limits the oxygen and nutrient supply to growing tumors as well as their metastatic capabilities. Withanolides demonstrate significant angiogenesis inhibition [55] and

as previously described, mechanisms include blocking NF-κB activity along with inhibition of cyclin D1 expression through suppressing proteasome-mediated ubiquitin degradation of IκBα [52, 55], and the interplay between NF-κB, JAK/STAT, HIF-1 and downstream targets VEGF, hnRNP-K, and MMPs [52, 140, 159]. Using both a subcutaneous flank xenograft and lung metastasis murine model with fibrosarcoma cells, withanone and WA demonstrated significant suppression of both subcutaneous and lung metastasis tumor growth compared to controls. The ex vivo tumors then demonstrated decreased expression of hnRNP-K and downstream effectors VEGF, Erk p44/42, and MMP2 [159]. In an in vivo assay of neovascularization using subcutaneously injected Matrigel that contained VEGF and bFGF, mice treated with 3-azido withaferin A (3-azidoWA) demonstrated a marked reduction in neovascularization with both preventative and treatment dosing when compared to untreated controls [50].

Not only have withanolides demonstrated significant cytotoxicity in multiple cancer models, but they have also demonstrated the capability to prevent cancer growth and implantation [137]. The induction of the phase II enzyme quinone reductase (QR) in mouse hepatoma cells has been used as a biological screen for chemopreventive compounds, and multiple withanolides have demonstrated robust induction of QR with minimal toxicity [191]. Subsequently in rodent models, pretreatment with the WS root isolate 1-oxo-5beta, 6beta-epoxy-witha-2-enolide prevented the formation of cutaneous malignancy and were absent p53 + foci that were noted in untreated rats that had been exposed to ultraviolet B radiation and benzoyl peroxide [192]. Additionally, WS root extract inhibited benzo(a) pyrene-induced forestomach papillomagenesis by 60 % and 7,12-dimethylbenzanthracene-induced skin papillomagenesis by 45 % [193]. It has been proposed that one mechanism of cutaneous chemoprevention is through inhibition of carcinogen-induced upregulation of acetyl-CoA carboxylase by suppressing AP-1 activation [194]. In a mouse mammary tumor virus-neu (MMTV-neu) transgenic model that forms spontaneous tumors, preventative WA treatment led to a significant 50 % reduction in average mammary tumor weight, a 95 % reduction in average area of invasive carcinoma, and a 73 % reduction in incidence of lung metastasis [195]. Another important antitumor effect of these withanolides is that they can also target breast cancer stem cells. Treatment of breast cancer cells with WA resulted in decreased ability to form mammospheres as well as significantly decreased aldehyde dehydrogenase activity within the mammary tumor cells [196]. WS root extract has also been shown to reduce the formation of spontaneous estrogen receptor negative mammary tumors in the MMTV-neu mice when mice were fed a diet containing the extract [197].

Overall these in vivo studies also noted that withanolides were well tolerated without significant reductions in animal weight, necrosis, or fibrosis when compared to placebo [23, 143, 159, 189, 190, 192, 193, 195]. The promising results of withanolides observed thus far in cancer models have led to ongoing research by many centers to both identify new withanolides and evaluate existing withanolides in multiple cancer models. In the last few years, withanolides have also been shown to demonstrate tumor cytotoxicity using animal xenografts in gliomas [198], B-cell

lymphomas [199], ovarian cancer [200, 201], prostate cancer [202, 203], soft tissue sarcoma [204], cervical carcinoma [205], melanoma, and mesothelioma [206]. These exciting results all point toward the incredible potential of these natural products for future clinical treatment regimens.

14.5.2 Clinical Applications of Withanolides for Cancer

Despite having improvements in our understanding of the multiple mechanisms of cytotoxicity with withanolide treatment across an expanding range of cancers, there has yet to be a significant human clinical placebo-controlled trial brought to completion and published that evaluates the efficacy of withanolides for the treatment of cancer. Withanolides are generally regarded as safe and have been used in human clinical trials for inflammatory and neurologic diseases, and have been evaluated for the treatment of fatigue for breast cancer patients undergoing chemotherapy [207]. Additional in vivo research on withanolides is ongoing and has overall been quite promising to identify withanolides as an important cancer therapy, however, further supporting research is needed to initiate the human clinical trials required to obtain a better understanding of the clinical treatment effects of withanolides on cancer. To date, there is no Food and Drug Administration (FDA)-approved good manufacturing process (GMP) facility currently purifying and producing purified withanolide compounds for use in clinical trials, although several facilities in other countries produce capsules of withanolide plant products and extracts that are not regulated by the FDA.

14.6 Application of Withanolides in Neurologic Diseases

14.6.1 Neurodegenerative Diseases

Neurodegenerative diseases are characterized by the progressive dysfunction and loss of neurons in the central nervous system (CNS) and are a major cause of dementia, cognitive and motor dysfunction. As the pathophysiology of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) are better understood, the major role of the immune system and neuroinflammation has become readily apparent [208]. Although the blood–brain barrier maintains some degree of separation of the CNS from the systemic immune system to aid in the immune privileged state of the CNS (its relative inability of nonself antigens to illicit an immune response), the role of the innate and adaptive immune responses has become a major focus of both study and intervention [209]. In addition to the formation and deposition of amyloid β plaques in AD, activation of microglia (macrophage-like CNS cell) and astrocytes,

increased complement components, cytokines, and TLR pathways, and alterations in peripheral Th cell responses have also been described [210, 211]. In PD, mitochondrial dysfunction, oxidative stress, and altered protein handling with Lewy body deposition as well as inflammation through microglial activation, increased IL-1β, IL-6, TNF-α, and TLRs, formation of antibodies to neuronal antigens, and Th cell modulation are important. Finally in HD, mutant Huntingtin (HTT) expression in neurons and glia leading to microglia proliferation/activation, increased complement 3, 9 and neuroinflammation are keys to the pathogenesis of the disease [208, 209].

As previously discussed, withanolides inhibit multiple inflammatory pathways, and within the CNS astrocytes, Withaferin A has been shown to attenuate LPS-induced NF-κB, TNF-α, COX-2 and iNOS [212]. In addition to neuroinflammatory modulation, several withanolides including WS extract, withanosides (particularly withanoside IV and its active metabolite sominone), the synthetic withanolidedenosomin, withanolide A and coagulin Q have all demonstrated important effects on stimulating neurite outgrowth and regeneration [66, 210, 211]. In AD, additional key findings demonstrated that withanolide A significantly downregulates beta-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1; known as β-secretase, enzyme involved in production of Aβ) while it upregulates a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10; α-secretase, non-amyloidogenic enzymatic processing of APP). Additionally, withanolide A increases expression of insulin-degrading enzyme, which is important in the proteolytic degradation of Aβ [213]. Multiple withanolides (WS, WA, and bracteosins) have also demonstrated significant acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) inhibition [214, 215] with computational docking analysis supporting withanolide A high-affinity binding for active sites on AChE [216]. These results, combined with the known antioxidant and anti-inflammatory effects of withanolides have propelled their research forward in neurodegenerative disorders to further elucidate their mechanism of action.

14.6.2 In Vivo Activities of Withanolides in the Treatment of Neurodegenerative Diseases

In a systematic review and meta-analysis, Durg et al. identified and analyzed 28 studies evaluating the use of WS in neurobehavioral disorders (including AD, PD, HD and anxiety/stress) induced by brain oxidation in rodent models [217]. Overall, WS treatment had a protective effect on brain oxidative stress that corrected abnormal activity levels of super oxide dismutase (SOD), catalase, glutathione peroxidase and glutathione, lipid peroxidation, and levels of nitrite and AChE [217]. In several types of AD mouse models, including Aβ-[25–35] induced memory deficit and 5XFAD mice (5 FAD mutations carried on APP and PS1 transgenes), withanolides (withanolide A, withanoside IV and its main active

metabolite, sominone, and WS extract) significantly ameliorated the impairment of spatial memory and behavioral deficits [39, 40, 210, 217], while ex vivo brains showed increased axonal and dendritic protein markers back to control levels [39, 211], increased axonal densities [40], and reduced Aβ levels and plaque depositions through upregulation of low-density lipoprotein receptor-related protein within the liver [218].

Parkinson's disease is characterized by an age-related neurodegenerative progression that leads to resting tremor, rigidity, and akinesia though loss of dopaminergic nigro-striatal neurons, particularly within the substantia nigra [219]. The rodent models of Parkinsonism are created through brain injection of 6-Hydroxydopamine [220] or systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [221] or the combination of manganese ethylene-bis-dithiocarbamate (maneb) and *N,N′*-dimethyl-4,4′-bipyridinium dichloride (paraquat) [222]. They cause specific degeneration and toxicity to catecholaminergic and dopaminergic neurons though oxidative stress and formation of hydrogen peroxide, hydroxyl, and superoxide radicals [220–222]. Using these models, treatment with WS extract resulted in significant improvements in locomotor control (including rotation, locomotion, muscular coordination and rearing), amelioration of oxidative parameters (including LPO, glutathione and associated enzymes, SOD and catalase), correction of ex vivo rodent brain alterations to catecholamine, dopamine, and dopamine metabolites (DOPAC/HVA) in ex vivo rat brains [222–226]. Some of these effects may be due to mediation of the otherwise proapototic state through reductions of Bax and Bcl-2 expression and neuroinflammatory astrocyte activation [227].

Huntington's disease has autosomal dominant transmission of the mutated HTT gene that leads to neuron destruction within the basal ganglia, leading to dementia and the characteristic involuntary writhing movements characteristic of Huntington's chorea. Mutant HTT aggregates have been implicated in oxidative stress through the formation of free radicals, which creates imbalance and plays a role in subsequent neuroinflammation [228]. Huntington's disease-like neurobehavioral and biochemical changes are induced in rodent models using neurotoxin 3-Nitropropionic acid. Treatment with WS root extract demonstrated significant dose-dependent attenuation of AChE levels, significantly improved oxidative stress markers, and improvement in general locomotor, performance and behavioral changes tested by Rotarod and Morris water [229, 230].

Although the involvement of the immune response and neuroinflammation plays a significant role in the pathogenesis of neurodegenerative diseases, the specific connections between these inflammatory pathways and the effects of withanolide treatment have not been well defined. The role of WS on reducing the oxidative stress within neurons has been demonstrated in multiple animal models of different disease processes, as well as the neuro-regenerative role of several withanolides on axonal and dendritic outgrowths and functional locomotor improvements. Connecting these effects to neuroinflammation and immunomodulation identifies an important area of future research.

14.6.3 Clinical Activity of Withanolides in Neurodegenerative Diseases

There is an overall paucity of research on the effects of withanolides in patients with neurodegenerative diseases. WS was a component of one study that evaluated the efficacy of a traditional Ayurveda treatment in clinically diagnosed PD patients. Treatment with a combination of eliminative cleansing and a concoction of cow's milk with powdered *Mucunapruriens*, *Hyoscyamusreticulatus* seeds, WS and *Sidacordifolia* roots (analyzed to contain 200 mg L-DOPA per dose) showed significant improvements in tremor/bradykinesia, stiffness and cramp-like pain resulting in improved activities of daily living, however, no changes in other symptoms like dysphonia, dysarthria, wasting, cogwheel rigidity, shuffling gait, or other locomotor symptoms [231]. However, conclusions regarding the role of WS cannot be reached due to the mixed nature of the L-DOPA containing herbal treatment. However, the growing in vitro and animal data continue to support the use of withanolides in neurodegenerative diseases, and illustrates the potential for its use in a more controlled clinical trial.

14.6.4 Withanolides in Neurobehavioral/Psychiatric Diseases

There is a broad range of neurological diseases in which inflammation and particularly cytokines and microglial activation have been shown to play a pathophysiological role. These include chronic stress [232], anxiety [233], depression [234], schizophrenia [235, 236], bipolar disorder [237, 238], and obsessive–compulsive disorder (OCD) [239]. Although the study of how the immune system and inflammation contribute to these diseases is relatively recent, major inflammatory pathways and cytokines have been identified. One proposed mechanism is through the stress response, whether through external stimuli or psychological imbalances, which leads to activation of the hypothalamic-pituitary-adrenal (HPA) axis and short-term elevations in glucocorticoids (mainly cortisol) and a subsequent anti-inflammatory response. However, prolonged stress and sustained HPA activation causes cortisol resistance and initiation of pro-inflammatory pathways, such Th cell release of IL-1α and β, IL-2, IL-6, IL-10, TNF-α cytokines, activation of NF-κB pathway, and microglial activation [232–238]. In addition to the known anti-inflammatory effects of withanolides previously discussed, they have also been described as adaptogens that assist with balance and regulation of the body's physiologic response to stressors [240]. The adaptogen mechanism of effect has been proposed to occur through modulation of the HPA axis, inducing stress-activated c-Jun N-terminal protein kinase (JNK1), inhibiting iNOS expression, and modulation of Hsp70 chaperone function [241]. Additionally, the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), which plays an important inhibitory role in neurological disorders, has

more recently been shown to have key anti-inflammatory interactions within the immune system through suppression of T-cells and macrophages, and inhibition of NF-κB on lymphocytes [242]. The GABA-mimetic activity of WS root extract has been shown for several decades [243] with a recent study identifying a 27 times greater affinity of WS for the highly sensitive GABAρ1 receptors compared to $GABA_A$ receptors, though WA and withanolide A were not the active GABA-mimetics [244]. These GABA-mimetic and adaptogenic effects likely play a significant role in the long-standing history of using Ashwagandha for the treatment of anxiety and neurobehavioral disorders, though the vast majority of animal and human studies have been performed in the last several decades.

14.6.5 In Vivo Activity of Withanolides for Neurobehavioral/Psychiatric Diseases

Most animal studies evaluating neurologic disorders have focused on the effects of WS extract using models of either chronic stress or withdrawal to induce anxiety and depression. Using a chronic, unpredictable, mild footshock to create a chronic stress model in rats, treatment with aqueous ethanol WS root extract significantly attenuated chronic, stress-induced abnormalities with improvements in biochemical imbalances (elevated blood glucose and corticosteroid levels), reduced number and severity of gastric ulcers, improved behavioral depression and sexual responses, improved cognitive memory function, and rescued immunosuppression in macrophage activity and immunologic pedal edema [245]. In depression and anxiety rodent models using chronic stress, isolation, sleep deprivation, WS extract treatment demonstrated significant antidepressant effects, and potentiated conventional antidepressants (tricyclic antidepressant imipramine and selective serotonin-reuptake inhibitor fluoxetine) measured by reduced immobility time in the forced open swim test ("behavioral despair/learned helplessness"), and an anxiolytic effect similar to benzodiazepines (lorazepam and diazepam) in the elevated plus maze, the social interaction test, and the feeding latency test [246–250].

In an OCD rodent model of marble-burying behavior, intraperitoneal injections of both methanolic and aqueous WS root extracts 30 min prior to evaluation resulted in significant dose-dependent reductions in marble-burying behavior similar to standard fluoxetine alone, and a synergistic effect in combination with fluoxetine [251]. These in vivo studies demonstrate a consistent amelioration of stress, anxiety, depressant, and OCD behaviors with corresponding corrections in biochemical abnormalities, however, the modulation of inflammatory pathways in these diseases requires further characterization.

14.6.6 Clinical Activities of Withanolides in Neurobehavioral/Psychiatric Diseases

Recent clinical trials using WS extract as a neurological treatment have evaluated effects on psychomotor function in patients with anxiety, bipolar disorder, and schizophrenia and provide clinical support for centuries of WS use in Ayurvedic medicine. There have been several randomized controlled trials that demonstrate significant improvement in anxiety and stress relief [252–257]. Studying ICD-10 anxiety disorders (generalized anxiety disorder, mixed anxiety and depression, panic disorder, and adjustment disorder with anxiety) assessed by Hamilton Anxiety Scale, patients were treated with 500 mg WS extract or placebo twice daily with subsequent clinically guided dose titrations. At 6 weeks, the WS extract treatment response of 88 % was significantly improved compared to the 50 % placebo response with no significant difference in overall adverse outcomes or with abrupt cessation of WS treatment [253]. Subsequent randomized controlled studies have corroborated their results and demonstrated either significantly decreased anxiety or improved stress relief using multiple anxiety scales (Hamilton Anxiety Scale, Beck Anxiety Inventory, Perceived Stress Scale), a range of WS dosing primarily between 125 and 600 mg/day for extract (one study 12,000 mg/day whole dried root powder) over the course of 60–84 days [252, 254–257]. None of the studies reported significant adverse events with WS treatment, with all described as mild or comparable to the placebo groups. However, significant limitations existed in each of the studies that included high rates of patient withdrawal from study [253], low sample size leading to bias risk and under powering, inconsistent dosing or lack of withanolide standardization, lack of comparison to standard of care anti-anxiolytics, and possible methodological flaws [252–257]. The limitations impair making definitive conclusions regarding the effectiveness of WS in anxiety disorders, but the body of study identifies WS as a relatively safe therapeutic and supports the role for conducting additional clinical studies.

In both bipolar disease and schizophrenia, cognitive impairments are consistently associated with poorer functional outcomes. As discussed previously, WS improves memory and cognitive function when treating neurodegenerative rodent models, and in healthy adults significantly improves psychomotor function as demonstrated by simple reaction times, choice discrimination, digit symbol substitution, digit vigilance, and card sorting tests compared to placebo [258]. In well maintained bipolar disease patients, a randomized controlled, trial of WS in adjunct to maintenance bipolar treatment resulted specific improvement in several cognitive function tests including the digit span backward, Flanker neutral response time, and social cognition response rating from the Penn Emotional Acuity Test, though no global cognitive improvement [259]. In schizophrenia, there is currently an ongoing randomized controlled clinical trial in the United States evaluating the effect of WS extract on symptom severity and associated stress that also aims to characterize

alterations in inflammatory cytokines [260]. The results from this study will help provide valuable insight into the effect of WS on neuroinflammation and schizophrenia symptoms.

14.7 Conclusions

Withanolides are an incredibly bio-diverse group of naturally occurring steroidal lactones. These plant-derived compounds have ongoing use within Ayurvedic medical practices and are important plant-derived medicinal compounds for a variety of human diseases and conditions. With WA as the archetype of this novel class drugs, there are now approximately 900 withanolides identified from a natural source or synthetically modified. Significant strides have been made in recent years to advance our understanding of the biochemical, immunomodulatory, and anti-inflammatory mechanisms of these compounds and plant extracts. Added to this is emerging in vivo and clinical evidence of safe, efficacious treatment effects across multiple disease processes ranging from autoimmune/inflammatory disorders, cancer, neurodegenerative diseases, and neurobehavioral/psychiatric diseases. Ongoing research on withanolides continues to identify new compounds and analogs, both plant derived and synthetically altered, that are more potent or specific for use in particular diseases. Given their impressive biologic activity in a number of challenging and complex disease processes and their unique ability to synergize with many standard drug treatments, these naturally derived drug compounds undoubtedly will have an important role clinically in well-designed combination strategies once disease-specific mechanisms of action and synergy are further validated and characterized. As such, they remain a very hot area of research as a novel group of medicinal therapeutics.

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