

RESEARCH MONOGRAPH . COMPOUND PROFILE

Withaferin-A

A C28-ergostane-type steroidal lactone isolated from Withania somnifera, distinguished by covalent multi-target pharmacology spanning NF-kappaB inhibition, vimentin intermediate filament disruption, Hsp90 C-terminal binding, and leptin sensitization, with preclinical anticancer efficacy across multiple tumor types and an emerging Phase I clinical safety record in advanced osteosarcoma.

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AUTHOR	Kodi ac biolabs Research
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ABSTRACT

Withaferin-A (WA), the prototypical withanolide and the first member of the C28-ergostane steroidal lactone class to be structurally characterized, is a naturally occurring phytochemical isolated principally from the leaves and roots of *Withania somnifera* (Linnaeus) Dunal (Solanaceae), a plant used for millennia in Ayurvedic medicine under the common name ashwagandha. The compound was first isolated by Lavie and colleagues in 1965 from the leaves of *Withania somnifera* and *Acnistus arborescens*, and its structural elucidation established the withanolide class as a distinct group of naturally occurring steroidal lactones characterized by a C28-ergostane skeleton with an oxidized delta-lactone side chain formed between carbons 22 and 26 [1, 2]. The chemical reactivity of Withaferin-A derives from three electrophilic pharmacophores: the alpha,beta-unsaturated ketone in the A ring, the 5beta,6beta-epoxide in the B ring, and the unsaturated delta-lactone in the side chain, each capable of forming covalent adducts with nucleophilic cysteine residues on target proteins through Michael addition chemistry [3].

The molecular pharmacology of Withaferin-A is characterized by covalent, multi-target engagement. The compound binds covalently to the sole cysteine residue (Cys328 in human vimentin) in the alpha-helical coiled-coil 2B domain of the intermediate filament protein vimentin, inducing filament aggregation, disrupting cytoskeletal architecture, and inhibiting epithelial-to-mesenchymal transition in cancer cells [4, 5]. It inhibits NF-kappaB signaling by direct thioalkylation of IKKbeta, preventing IkappaB phosphorylation and nuclear translocation of the NF-

kappaB p65/p50 heterodimer [6, 7]. It binds the C-terminal domain of Hsp90 through an ATP-independent mechanism, inducing proteasomal degradation of Hsp90 client proteins including Akt, Cdk4, and the glucocorticoid receptor [8]. Additional validated direct targets include annexin II, beta-tubulin, mortalin (mitochondrial Hsp70), KEAP1, and Bruton tyrosine kinase [3, 9]. The breadth of covalent target engagement produces convergent antiproliferative, pro-apoptotic, anti-angiogenic, and anti-inflammatory activity across multiple cancer cell lines and xenograft models.

Preclinical anticancer efficacy has been demonstrated in xenograft and orthotopic tumor models of breast cancer (including triple-negative breast cancer), ovarian cancer, cervical carcinoma, glioblastoma, pancreatic cancer, melanoma, neuroblastoma, and multiple myeloma, with tumor growth inhibition typically observed at intraperitoneal doses of 2 to 8 mg/kg in mice [10, 11, 12]. Beyond oncology, Withaferin-A has been characterized as a leptin sensitizer with strong antidiabetic and anti-obesity properties; treatment of diet-induced obese mice produced 20 to 25 percent reduction in body weight, reversal of hepatic steatosis, and normalization of glucose metabolism independently of the leptin-sensitizing effect [13]. Neuroprotective activity has been demonstrated in multiple rodent models.

Pharmacokinetics in rodents are characterized by low oral bioavailability (approximately 1.8 to 32 percent depending on formulation and species), rapid hepatic metabolism, and a short plasma half-life of approximately 1.36 hours in mice [14, 15]. The compound crosses the blood-brain barrier. The LD50 following oral administration in mice exceeds 2000 mg/kg body weight, placing Withaferin-A in GHS toxicity category 5 [16]. A Phase I

dose-escalation clinical trial in patients with advanced-stage high-grade osteosarcoma (Pires et al. 2019) evaluated oral doses of 72, 108, 144, and 216 mg of Withaferin-A per day and reported tolerability without dose-limiting toxicity; adverse events were limited to grade 1 and grade 2 severity, principally liver enzyme elevation and skin rash [17]. The maximum tolerated dose was not reached. Circulating Withaferin-A could not be quantified by the HPLC bioanalytical method employed, consistent with the low oral bioavailability observed in preclinical studies and representing a principal translational challenge. This monograph reviews the chemistry, isolation, and biosynthesis of Withaferin-A; the covalent multi-target pharmacology; the pharmacokinetic record; the preclinical efficacy across oncology, metabolic, and neurological models; the Phase I clinical evidence; sourcing and quality verification; reconstitution and handling; stack-interaction considerations; adverse-event signal; and a comparative assessment of five natural-product anticancer and anti-inflammatory candidates against Withaferin-A on five competency standards.

1. Compound Identification

1.1 Names, synonyms, and registry identifiers

Systematic name: Withaferin-A.

Common synonyms: Withaferin A, WA, WFA, NSC-101088.

Plant source: *Withania somnifera* (Linnaeus) Dunal (ashwagandha, Indian winter cherry, Indian ginseng); also isolated from *Acnistus arborescens*, *Dunalia australis*, and other Solanaceae family members.

Chemical Abstracts Service registry number: 5119-48-2.

PubChem Compound Identifier: CID 265237.

International Union of Pure and Applied Chemistry name: (4 β ,5 β ,6 β ,22R)-4,27-dihydroxy-5,6:22,26-diepoxyergosta-2,24-dien-1,26-dione. The compound is a 28-carbon ergostane-type steroidal lactone carrying a 2-en-1-one A ring, a 5 β ,6 β -epoxide in the B ring, and a delta-lactone formed between carbons 22 and 26 in the side chain [1, 2].

Compound class: Withanolide (C28-ergostane steroidal lactone). Withaferin-A is the founding and most extensively studied member of the withanolide class, a group of approximately 300 naturally occurring steroidal lactones isolated principally from Solanaceae family plants [18].

1.2 Structural class and chemistry

Withaferin-A belongs to the withanolide subclass of ergostane-type steroidal lactones. The structural skeleton comprises four fused rings (three cyclohexane rings designated A, B, and C, and one cyclopentane ring designated D) with an oxidized side chain forming a six-membered delta-lactone between carbons 22 and 26 [1].

Molecular formula: C₂₈H₃₈O₆.

Exact mass: 470.2668 g/mol (monoisotopic).

Molecular weight: 470.60 g/mol.

Physical properties: White to off-white crystalline powder. Soluble in dimethyl sulfoxide (DMSO), methanol, ethanol, and chloroform; poorly soluble in water. Melting point approximately 252 to 253 degrees Celsius. Optical rotation $[\alpha]_D$ approximately +125 degrees ($c = 1.0$, chloroform).

Reactive pharmacophores: The biological activity of Withaferin-A is attributed to three electrophilic centers, each capable of forming Michael addition adducts with nucleophilic thiol groups on cysteine residues in target proteins [3]. These are: (a) the α,β -unsaturated ketone (2-en-1-one) in the A ring, the most reactive electrophilic center and the primary site of covalent target engagement; (b) the $5\beta,6\beta$ -epoxide in the B ring, which undergoes ring-opening nucleophilic attack; and (c) the unsaturated δ -lactone in the side chain (C22-C26). Structure-activity relationship studies have demonstrated that the A-ring unsaturated ketone is necessary for anticancer and NF- κ B inhibitory activity; reduction or saturation of this pharmacophore substantially diminishes biological potency [19].

Stereochemistry: Withaferin-A possesses six stereocenters (at carbons 4, 5, 6, 10, 13, and 22) and is isolated as a single enantiomer. The natural product has the (4 β ,5 β ,6 β ,22R) configuration. Synthetic modification of stereochemistry has been used in structure-activity relationship studies but the natural configuration is the biologically active form.

1.3 Isolation and biosynthesis

Withaferin-A is isolated from the leaves, roots, and berries of *Withania somnifera* by organic solvent extraction (typically methanol or ethanol), followed by liquid-liquid partitioning, silica gel column chromatography, and preparative high-performance liquid chromatography [18]. Yields from dried leaf material are typically 0.01 to 0.1 percent by weight, depending on the plant chemotype, cultivation conditions, and harvest timing.

The biosynthetic pathway proceeds through the mevalonate pathway to produce 24-methylenecholesterol, which undergoes cytochrome P450-mediated oxidations at multiple positions to introduce the 1-oxo-2-ene functionality in the A ring, the $5,6$ -epoxide, the 4-hydroxyl, and the side-chain oxidations that form the δ -lactone [20]. The pathway has been partially elucidated through isotope labeling and transcriptomic studies, but key oxidative enzymes remain uncharacterized.

Total synthesis of Withaferin-A has been achieved, but the complexity of the molecule (six stereocenters, three reactive functional groups) renders total synthesis impractical for bulk supply. Semi-synthetic modification of naturally sourced Withaferin-A is the principal route to structural analogs. Research-grade and commercial supply is therefore predominantly through extraction from cultivated *Withania somnifera* plant material.

2. Abstract

Withaferin-A is a steroidal lactone of the withanolide class, isolated from *Withania somnifera* and related Solanaceae plants, that has emerged over the past two decades as one of the most extensively characterized multi-target natural-product anticancer and anti-inflammatory agents in preclinical research. The compound exerts its biological effects through covalent modification of multiple protein targets via Michael addition chemistry at its three electrophilic pharmacophores, producing convergent antiproliferative, pro-apoptotic, anti-angiogenic, anti-inflammatory, and metabolic effects that have been validated across numerous cancer cell lines, xenograft tumor models, and disease-relevant animal models [3, 10].

The principal validated molecular targets are: (a) vimentin, where covalent modification of Cys328 in the 2B coiled-coil domain induces intermediate filament aggregation and inhibits epithelial-to-mesenchymal transition [4, 5]; (b) IKKbeta, where thioalkylation prevents NF-kappaB nuclear translocation and suppresses inflammatory cytokine expression [6, 7]; (c) Hsp90, where C-terminal binding disrupts chaperone function and induces proteasomal degradation of oncogenic client proteins [8]; and (d) annexin II, beta-tubulin, mortalin, KEAP1, and Bruton tyrosine kinase through additional covalent interactions [3, 9]. The multi-target covalent pharmacology distinguishes Withaferin-A from single-target synthetic anticancer agents and from other natural-product NF-kappaB inhibitors.

Preclinical anticancer efficacy spans breast cancer (including triple-negative breast cancer), ovarian cancer, cervical carcinoma, pancreatic cancer, glioblastoma, melanoma, colon cancer, multiple myeloma, and osteosarcoma in both in vitro and in vivo models [10, 11, 12]. In xenograft studies, Withaferin-A at intraperitoneal doses of 2 to 8 mg/kg typically produces 50 to 70 percent tumor growth inhibition relative to vehicle controls. The compound additionally suppresses tumor angiogenesis and inhibits metastatic colonization in experimental metastasis models. Beyond oncology, Withaferin-A functions as a leptin sensitizer with antidiabetic and anti-obesity properties in diet-induced obese mice [13], promotes white adipose tissue browning through the sympathetic nerve-activated Prdm16-FATP1 axis [21], and demonstrates neuroprotective activity in models of neurodegeneration and cerebral hemorrhage [22].

The Phase I clinical trial in advanced-stage high-grade osteosarcoma (Pires et al. 2019) established preliminary human safety at oral doses up to 216 mg per day without dose-limiting toxicity [17]. Clinical translation is limited by low oral bioavailability (approximately 1.8 percent in rats), rapid hepatic metabolism, and the inability to detect circulating Withaferin-A by conventional HPLC methods at clinically administered doses [14, 17]. Formulation strategies including liposomal encapsulation, nanoparticle delivery, and prodrug design are active areas of investigation.

This monograph provides a comprehensive review of Withaferin-A spanning compound identification, discovery history, multi-target molecular pharmacology, pharmacokinetics, preclinical efficacy, clinical evidence, sourcing, handling, drug interactions, safety, and comparative assessment against five alternative natural-product anticancer candidates.

3. Discovery and Development History

3.1 Isolation and structural elucidation

Withaferin-A was first isolated in 1965 by David Lavie and colleagues at the Weizmann Institute of Science in Rehovot, Israel, from the leaves of *Withania somnifera* collected in Israel [1]. The isolation was part of a systematic phytochemical investigation of Solanaceae plants with reported medicinal properties. Lavie and colleagues established the molecular formula ($C_{28}H_{38}O_6$), the steroidal skeleton, and the principal functional groups through degradation studies, infrared spectroscopy, and nuclear magnetic resonance spectroscopy. The complete stereostructure was published in a series of papers in the Journal of the Chemical Society between 1965 and 1966 [1, 2]. An independent isolation from *Acnistus arborescens* leaves was reported contemporaneously.

The structural novelty of Withaferin-A, specifically the 22,26-delta-lactone side chain on an ergostane skeleton, prompted Lavie to designate the class "withanolides" after the genus *Withania*. Subsequent phytochemical work over the following decades identified approximately 300 withanolide structures across multiple Solanaceae genera, establishing the withanolides as one of the largest classes of plant-derived steroidal natural products [18].

3.2 Early biological characterization

Early biological studies in the 1960s and 1970s identified Withaferin-A as possessing antitumor activity against Ehrlich ascites carcinoma in mice and as an immunosuppressive agent [23]. The National Cancer Institute (NCI) assigned the compound the identifier NSC-101088 and included it in screening programs. However, the absence of a defined molecular target and the complexity of the natural-product pharmacology limited development interest during the era of target-directed drug discovery.

3.3 Modern target identification

The molecular pharmacology of Withaferin-A was substantially elucidated beginning in the early 2000s through three landmark studies. Falsey et al. (2006) identified vimentin as a direct binding target using biotinylated Withaferin-A affinity probes and demonstrated that covalent modification of vimentin Cys328 induced intermediate filament aggregation and antiangiogenic activity [4]. Yu et al. (2010) characterized Hsp90 C-terminal binding and the downstream degradation of Hsp90 client proteins in pancreatic cancer cells [8]. Kaileh et al. (2007) characterized the mechanism of NF-kappaB inhibition through direct IKKbeta thioalkylation [7]. These three studies collectively established the multi-target covalent pharmacology that defines the current understanding of Withaferin-A mechanism of action.

3.4 Expansion of preclinical and clinical investigation

Between 2010 and 2025, the research literature on Withaferin-A expanded substantially, with published preclinical efficacy data in more than 15 cancer types, the identification of additional molecular targets (annexin II, beta-tubulin, KEAP1, mortalin, Bruton tyrosine kinase), the characterization of metabolic and neuroprotective activities, and the completion of the first Phase I clinical trial in osteosarcoma [17]. A combination therapy clinical trial of liposomal doxorubicin with Withaferin-A in recurrent ovarian cancer has been reported in development. The compound remains in early clinical investigation; no registration trial has been completed and no regulatory approval has been obtained in any jurisdiction. The current research state is one of extensive preclinical validation across multiple disease areas, a preliminary human safety record, and substantial translational challenges related to oral bioavailability and formulation.

4. Mechanism of Action

Withaferin-A is a covalent, multi-target ligand. Its biological effects derive from Michael addition reactions between the three electrophilic pharmacophores (A-ring enone, B-ring epoxide, side-chain lactone) and nucleophilic cysteine residues on target proteins. The covalent binding is irreversible under physiological conditions and produces sustained target inhibition that outlasts the plasma residence of the free compound.

4.1 Vimentin targeting and intermediate filament disruption

Vimentin is a type III intermediate filament protein expressed in mesenchymal cells, in cancer cells undergoing epithelial-to-mesenchymal transition (EMT), and in activated immune cells. Withaferin-A binds covalently to Cys328 (human numbering; Cys327 in some species), the sole cysteine residue in the highly conserved alpha-helical coiled-coil 2B rod domain of vimentin [4]. This cysteine is positioned at the interface between vimentin dimers in the coiled-coil assembly, and its covalent modification by Withaferin-A disrupts the normal lateral and longitudinal assembly of vimentin filaments.

The functional consequences of vimentin targeting include: aggregation of vimentin intermediate filaments into punctate cytoplasmic aggregates that colocalize with F-actin [4]; inhibition of EMT in breast cancer and non-small cell lung cancer cells, with suppression of mesenchymal markers (N-cadherin, vimentin itself, fibronectin) and restoration of epithelial markers (E-cadherin) [5]; inhibition of cancer cell migration and invasion in Boyden chamber and wound-healing assays; and antiangiogenic activity through disruption of endothelial cell intermediate filament networks.

The vimentin-targeting mechanism is particularly relevant to triple-negative breast cancer, which characteristically overexpresses vimentin as a marker of the mesenchymal phenotype. Thaiparambil et al. (2011) demonstrated that Withaferin-A suppressed vimentin protein levels in vivo in MDA-MB-231 xenograft tumors and inhibited experimental lung metastasis [5].

4.2 NF-kappaB pathway inhibition

The NF-kappaB signaling pathway is a master regulator of inflammation, innate immunity, and cell survival. In cancer, constitutive NF-kappaB activation promotes proliferation, survival, angiogenesis, and resistance to apoptosis. Withaferin-A inhibits NF-kappaB signaling at the level of the IKK complex through direct covalent modification of IKKbeta [6, 7].

The mechanism, characterized by Kaileh et al. (2007), involves thioalkylation of a reactive cysteine residue in the activation loop of IKKbeta, preventing its kinase activity and thereby blocking phosphorylation of IkappaBalpha [7]. In the absence of IkappaBalpha phosphorylation, the inhibitory protein is not ubiquitinated and degraded, and the NF-kappaB p65/p50 heterodimer remains sequestered in the cytoplasm. The downstream consequences include suppression of NF-kappaB-dependent gene expression, including tumor necrosis factor alpha, interleukin-1 beta, interleukin-6, interleukin-8, cyclooxygenase-2, matrix metalloproteinases (MMP-2, MMP-9), vascular endothelial growth factor, and anti-apoptotic proteins (Bcl-2, Bcl-xL, cIAP1, cIAP2, survivin) [6].

Oh et al. (2009) further demonstrated that Withaferin-A inhibits TNF-alpha-induced expression of cell adhesion molecules (ICAM-1, VCAM-1, E-selectin) in human pulmonary epithelial cells through NF-kappaB and Akt inactivation, establishing the anti-inflammatory dimension of this mechanism [24].

4.3 Hsp90 chaperone inhibition

Heat shock protein 90 (Hsp90) is a molecular chaperone essential for the conformational maturation and stability of numerous oncogenic client proteins, including Akt, Cdk4, HER2, EGFR, mutant p53, and the glucocorticoid receptor. Yu et al. (2010) demonstrated that Withaferin-A binds to the C-terminal domain of Hsp90 (distinct from the N-terminal ATP-binding site targeted by geldanamycin-class inhibitors) through an ATP-independent mechanism [8].

The C-terminal binding disrupts the Hsp90 chaperone cycle and induces proteasomal degradation of client proteins. In pancreatic cancer cells (Panc-1, MiaPaCa2), Withaferin-A treatment produced dose-dependent degradation of Akt, Cdk4, and the glucocorticoid receptor, effects that were reversed by the proteasome inhibitor MG132, confirming the proteasome-dependent mechanism [8]. The C-terminal

Hsp90 binding mechanism is complementary to N-terminal Hsp90 inhibitors and may offer therapeutic advantages in contexts where N-terminal inhibitors induce compensatory heat shock factor 1 activation.

4.4 Additional molecular targets

Proteomic and affinity-probe studies have identified additional direct binding targets of Withaferin-A:

Annexin II: Withaferin-A covalently modifies annexin II, a flexible adaptor protein involved in actin cytoskeletal organization, membrane dynamics, and cell motility. The consequence is actin microfilament aggregation and disruption of cell migration [9].

Beta-tubulin: Covalent binding to beta-tubulin disrupts microtubule polymerization dynamics, contributing to mitotic arrest and the antiproliferative activity of Withaferin-A [3].

KEAP1: Withaferin-A modifies cysteine residues on Kelch-like ECH-associated protein 1 (KEAP1), the cytoplasmic repressor of the Nrf2 transcription factor. KEAP1 modification releases Nrf2 for nuclear translocation and activation of antioxidant response element-driven gene expression, including heme oxygenase-1, NAD(P)H quinone dehydrogenase 1, and glutathione S-transferases [3].

Bruton tyrosine kinase (BTK): Covalent modification of BTK has been reported in proteomic studies, with potential implications for B-cell malignancy applications [3].

Mortalin (mitochondrial Hsp70): Interaction with mortalin has been implicated in the pro-apoptotic activity of Withaferin-A through disruption of mitochondrial function [3].

4.5 Composite pharmacology

The composite pharmacological profile of Withaferin-A integrates multiple convergent mechanisms that collectively produce anticancer, anti-inflammatory, anti-angiogenic, and metabolic effects. The NF-kappaB inhibition and Hsp90 client protein degradation suppress cancer cell survival signaling. The vimentin disruption inhibits EMT and metastasis. The annexin II and beta-tubulin targeting disrupts cytoskeletal dynamics and cell division. The KEAP1 modification activates cytoprotec-

tive gene expression in non-malignant cells. This convergent multi-target pharmacology distinguishes Withaferin-A from single-target anticancer agents and provides a mechanistic rationale for the broad-spectrum preclinical efficacy observed across diverse cancer types.

The multi-target mechanism also implies a relatively narrow therapeutic index, as the same covalent chemistry that produces anticancer activity can engage off-target proteins at higher concentrations. The pharmacological challenge is to achieve sufficient target engagement at the tumor site while maintaining systemic concentrations below the threshold for off-target toxicity.

5. Pharmacokinetics and Routes of Administration

5.1 Absorption

Oral bioavailability of Withaferin-A is low. Dai et al. (2019) characterized the oral pharmacokinetics of Withaferin-A in rats using LC-MS/MS and Q-TRAP methods and reported an oral bioavailability of approximately 32.4 percent [14]. A separate assessment by Patil et al. (2013) reported bioavailability of approximately 1.8 percent [15]. The discrepancy likely reflects differences in formulation, animal strain, dose, and analytical methodology. The consensus from multiple preclinical studies is that oral bioavailability is low, primarily due to extensive first-pass hepatic metabolism and poor aqueous solubility.

Dai et al. (2019) further demonstrated by rat intestine-liver in situ perfusion that Withaferin-A concentration decreased rapidly during hepatic first pass, with only 27.1 percent of the parent compound remaining after one hour of perfusion [14]. Withaferin-A was detectable in plasma up to 10 hours post-dose, suggesting a combination of absorption from the gastrointestinal tract and redistribution from tissue depots.

5.2 Distribution

Withaferin-A is highly lipophilic and distributes extensively into tissues. The compound crosses the blood-brain barrier, as evidenced by neuroprotective and neurological effects observed in multiple rodent studies [22]. Volume of distribution data in humans are not available. In mice, tissue distribution studies have demonstrated accumulation in liver, kidney, lung, and adipose tissue.

Plasma protein binding has not been formally characterized by equilibrium dialysis in published studies. The high lipophilicity (cLogP approximately 3.0 to 3.5) predicts substantial protein binding.

5.3 Metabolism

Withaferin-A undergoes extensive hepatic metabolism. Stability studies in liver microsomes demonstrate rapid degradation, with a half-life of approximately 5.6 minutes in both rat and human liver microsomes [14]. The metabolic pathways include cytochrome P450-mediated oxidation, epoxide hydrolysis, and glucuronide conjugation.

The specific CYP isoforms responsible for Withaferin-A metabolism have not been fully characterized. The rapidity of microsomal degradation suggests involvement of high-capacity hepatic CYP isoforms (potentially CYP3A4, CYP2C, and CYP1A families), though definitive isoform identification with selective inhibitors and recombinant CYP enzymes has not been published.

Intestinal metabolism also contributes to first-pass elimination. Withaferin-A was unstable in simulated gastric fluid and in intestinal microflora solutions, indicating that both chemical and microbial degradation reduce the fraction of orally administered compound reaching the systemic circulation [14].

5.4 Elimination

In mice, the plasma elimination half-life after intraperitoneal administration is approximately 1.36 hours [16]. The elimination half-life after oral administration is longer (reflecting ongoing absorption), with detectable plasma concentrations persisting to approximately 10 hours. Formal renal and biliary excretion studies have not been published. Given the extensive hepatic metabolism, elimination is predominantly metabolic rather than renal.

5.5 Routes of administration

Intraperitoneal (preclinical): The most common route in animal studies. Doses of 2 to 8 mg/kg are standard in xenograft and disease-model studies, typically administered in a vehicle of DMSO/PEG300/Tween 80/saline or DMSO/corn oil [10, 11].

Oral (preclinical and clinical): Used in pharmacokinetic studies and in the Phase I clinical trial. Oral doses in the Phase I trial ranged from 72 to 216 mg per day, administered as capsules containing a *Withania somnifera* extract standardized for Withaferin-A content [17].

Intravenous (preclinical): Used in some pharmacokinetic characterization studies. Requires solubilization in DMSO-containing vehicles.

5.6 Formulation challenges and strategies

The low oral bioavailability, poor aqueous solubility, and rapid hepatic metabolism of Withaferin-A represent the principal translational barriers. Active formulation strategies under investigation include: liposomal encapsulation to improve oral absorption and tumor delivery; polymeric nanoparticle formulations (PLGA, chitosan) to enhance bioavailability; cyclodextrin inclusion complexes to improve aqueous solubility; and prodrug approaches that mask the reactive A-ring pharmacophore during absorption and release the active compound at the target site. None of these advanced formulations has reached clinical evaluation as of the most recent monograph revision.

6. Preclinical Pharmacology

6.1 Anticancer activity in vitro

Withaferin-A demonstrates antiproliferative activity across a broad panel of human cancer cell lines. IC50 values are typically in the low micromolar range (0.5 to 5 micromolar) for 48- to 72-hour cytotoxicity assays [10]. Reported IC50 values include: MCF-7 breast cancer (1.2 to 2.5 micromolar), MDA-MB-231 triple-negative breast cancer (1.0 to 3.0 micromolar), Panc-1 pancreatic cancer (1.5 to 3.0 micromolar), HeLa cervical carcinoma (1.0 to 2.0 micromolar), U251 glioblastoma (2.0 to 4.0 micromolar), and A375 melanoma (1.0 to 2.5 micromolar).

The mechanisms of cytotoxicity involve: G2/M cell cycle arrest through beta-tubulin targeting and disruption of mitotic spindle assembly; induction of intrinsic (mitochondrial) apoptosis through caspase-3 and caspase-9 activation, cytochrome c release, and Bcl-2 family protein modulation; induction of endoplasmic reticulum stress through the ATF4-ATF3-CHOP axis (particularly characterized in glioblastoma cells); autophagy induction through mTOR pathway inhibition; and reactive oxygen species generation [10, 12].

Notably, Withaferin-A demonstrates selectivity for cancer cells over non-transformed cells in several comparative studies, with IC50 values in non-malignant cell lines typically 3- to 10-fold higher than in matched cancer cell lines. The selectivity is attributed in part to the higher vimentin expression in cancer cells (particularly those undergoing EMT) and the greater dependence of cancer cells on NF-kappaB and Hsp90 signaling for survival.

6.2 Anticancer activity in vivo

In vivo anticancer efficacy has been demonstrated in multiple xenograft models:

Breast cancer: Withaferin-A at 4 to 8 mg/kg intraperitoneal, three times weekly, produced 50 to 70 percent reduction in tumor volume in MDA-MB-231 xenografts [5, 11]. Stan et al. (2008) reported inhibition of MDA-MB-231 xenograft growth and induction of apoptosis at pharmacologically achievable doses [25]. Lee et al. (2016)

demonstrated that Withaferin-A inhibited *in vivo* growth of breast cancer cells accelerated by Notch2 knockdown, with significant suppression of xenograft tumor weight and volume at 20 mg/kg [26].

Ovarian cancer: Withaferin-A treatment of mice bearing ovarian tumors reversed cancer-associated cachexia and reduced expression of inflammatory cytokines in kidney tissue [27]. Combination studies with standard chemotherapy are in preclinical and early clinical development.

Pancreatic cancer: The Hsp90 inhibition mechanism (Yu et al. 2010) was characterized in pancreatic cancer models, with demonstration of Hsp90 client protein degradation *in vivo* [8].

Glioblastoma: Withaferin-A induced endoplasmic reticulum stress and apoptosis in glioblastoma cell lines and xenografts through the ATF4-ATF3-CHOP axis [12].

6.3 Metabolic and anti-obesity activity

Lee et al. (2016, *Nature Medicine*) characterized Withaferin-A as a leptin sensitizer with strong antidiabetic properties in mice [13]. In diet-induced obese mice, Withaferin-A treatment (intraperitoneal, daily dosing over several weeks) produced: 20 to 25 percent reduction in body weight; reversal of hepatic steatosis; improved glucose tolerance and insulin sensitivity; and reduction of obesity-associated metabolic abnormalities. The effect on glucose metabolism was independent of the leptin-sensitizing mechanism, indicating multiple metabolic targets. The leptin-sensitizing effect was demonstrated by showing that co-administration of Withaferin-A and leptin reduced food intake by 71 percent compared to vehicle plus leptin in leptin-resistant obese mice.

Kang et al. (2022, *Diabetes*) further demonstrated that Withaferin-A promotes white adipose tissue browning and prevents obesity through the sympathetic nerve-activated Prdm16-FATP1 signaling axis, inducing elevated energy expenditure and decreased respiratory exchange ratio [21].

6.4 Neuroprotective activity

Withaferin-A has demonstrated neuroprotective activity in several rodent models. The compound crosses the blood-brain barrier and has been characterized as reducing neuroinflammation, oxidative stress, and neuronal apoptosis in models of

neurodegeneration [22]. Combination with leptin has shown enhanced neurogenesis and reduced neurological deficit after intracerebral hemorrhage through activation of the STAT3/SOCS3 pathway. The neuroprotective literature is preclinical and has not produced clinical evidence. The low oral bioavailability and rapid metabolism represent barriers to neurological applications that may require sustained central nervous system exposure.

6.5 Anti-inflammatory activity

Beyond NF-kappaB inhibition, Withaferin-A suppresses multiple inflammatory mediators in preclinical models, including cyclooxygenase-2, inducible nitric oxide synthase, tumor necrosis factor alpha, interleukin-1 beta, and interleukin-6. Anti-inflammatory efficacy has been demonstrated in models of sepsis, acute lung injury, colitis, and arthritis. The anti-inflammatory mechanism is convergent with the anticancer mechanism through shared NF-kappaB pathway inhibition.

7. Clinical Evidence Base

7.1 Phase I trial in osteosarcoma

The sole published clinical trial of purified Withaferin-A is the Pires et al. (2019) Phase I dose-escalation study in patients with advanced-stage high-grade osteosarcoma [17]. The study enrolled 13 patients and employed a classical 3+3 dose-escalation design with four cohorts corresponding to daily Withaferin-A doses of 72, 108, 144, and 216 mg, administered as oral capsules containing a *Withania somnifera* extract standardized for Withaferin-A content, dosed as 2 capsules twice daily, 3 capsules twice daily, 4 capsules twice daily, and 4 capsules three times daily, respectively.

Safety: The formulation was well tolerated at all dose levels. Eleven adverse events of grade 1 or grade 2 severity were observed in 8 of 13 patients. No grade 3 or grade 4 adverse events were reported. The most common adverse events were elevation of liver enzymes (5 of 11 events) and skin rash (2 of 11 events). Other adverse events included fatigue, fever, edema, and diarrhea (one event each). No dose-limiting toxicity was observed at any cohort, and the maximum tolerated dose was not reached [17].

Pharmacokinetics: A significant limitation of the trial was that circulating Withaferin-A could not be quantified by the HPLC bioanalytical method employed. This finding is consistent with the low oral bioavailability (approximately 1.8 percent) observed in preclinical pharmacokinetic studies and represents a principal challenge for dose-response characterization and clinical development. More sensitive LC-MS/MS bioanalytical methods would be required for future pharmacokinetic characterization in humans.

Efficacy: The Phase I trial was designed for safety and pharmacokinetic assessment, not efficacy. Tumor response data were not formally reported as primary or secondary endpoints. The trial established preliminary human safety and tolerability, supporting further clinical investigation with improved formulations and more sensitive bioanalytical methods.

7.2 Ashwagandha extract clinical studies

Numerous clinical trials have evaluated *Withania somnifera* (ashwagandha) root and leaf extracts for stress, anxiety, cognitive function, and athletic performance endpoints. These extracts contain varying amounts of Withaferin-A (typically 1 to 5 percent of total withanolides) along with other withanolides (withanoside IV, withanoside V, withanolide A, withanolide B, 12-deoxywithastramonolide) and non-withanolide constituents. The clinical results from whole-extract studies cannot be attributed specifically to Withaferin-A, as the extract is a complex mixture. However, these studies provide indirect evidence for the safety of Withaferin-A exposure at the doses contained within the extract formulations.

A double-blind crossover bioequivalence study of ashwagandha extracts in healthy adults characterized the pharmacokinetics of withanolide glycosides and provided evidence for oral absorption of withanolide components from standardized extract formulations [28].

7.3 Clinical development pipeline

A Phase I/II clinical trial of combination therapy with liposomal doxorubicin and Withaferin-A in recurrent ovarian cancer has been reported in development, intended to assess feasibility and tolerance in Phase I and treatment response in Phase II. Published results from this trial are not available as of the most recent monograph revision.

The clinical-development state of Withaferin-A is therefore early Phase I, with a single published safety study in osteosarcoma, no efficacy data from controlled trials of purified Withaferin-A, and a combination trial in ovarian cancer in progress. The translational pathway is contingent on resolution of the oral bioavailability limitation and development of bioanalytical methods capable of quantifying circulating Withaferin-A at clinical doses.

8. Sourcing and Quality Verification

Withaferin-A is commercially available from multiple chemical and natural-product suppliers at research grade. Principal suppliers include Sigma-Aldrich/MilliporeSigma (catalog 681535), Selleckchem (S858702), MedChemExpress (HY-N2065), Cayman Chemical, Tocris Bioscience (catalog 2816), Abcam (ab120644), AdipoGen (AG-CN2-0490), Fermentek, LKT Laboratories, and BOC Sciences. Purity specifications are typically greater than 95 percent by HPLC, with premium lots available at greater than 98 percent.

Supply is predominantly through extraction from cultivated *Withania somnifera* plant material, principally from Indian and Israeli sources. Pricing varies with purity and quantity; typical research-grade pricing is approximately \$50 to \$150 per 10 milligrams at greater than 98 percent purity.

Investigators should confirm compound identity and purity on every research-grade lot through:

- High-performance liquid chromatography area-percent purity (C18 reverse-phase; UV detection at 227 nm), ideally greater than 95 percent for screening and greater than 98 percent for mechanism-of-action studies
- Mass spectrometry confirmation of molecular weight (471.27 [M+H]⁺ for the protonated molecular ion)
- Optical rotation measurement ([α]_D approximately +125 degrees in chloroform) to confirm the natural-product configuration
- Nuclear magnetic resonance spectroscopy (1H and 13C) for definitive structural confirmation, particularly to distinguish Withaferin-A from structurally similar withanolides (withanolide A, withanone, 27-deoxyWithaferin-A)
- Residual solvent analysis if the compound was purified by chromatography

Third-party analytical confirmation is recommended for any investigation where dose-response precision or target-engagement quantification is critical, given the natural-product origin and the potential for lot-to-lot variability in extraction-derived material.

9. Reconstitution and Handling

Withaferin-A is supplied as a white to off-white crystalline powder. The compound is poorly soluble in water and should not be reconstituted directly in aqueous vehicles.

Stock solution preparation: Dissolve in anhydrous DMSO at concentrations of 10 to 50 mg/mL (approximately 21 to 106 mM). Vortex or sonicate briefly until fully dissolved. Aliquot into single-use volumes to avoid repeated freeze-thaw cycles.

Storage: Store the solid compound at minus 20 degrees Celsius, protected from light and moisture, under desiccated conditions. DMSO stock solutions are stable for up to 2 years at minus 80 degrees Celsius and up to 1 year at minus 20 degrees Celsius, with protection from light [29].

Working solutions for in vitro studies: Dilute DMSO stock solutions into culture medium immediately before use. The final DMSO concentration should not exceed 0.1 percent (v/v) to avoid solvent-mediated cytotoxicity. For dose-response studies, prepare serial dilutions in DMSO first, then dilute into medium to maintain constant DMSO concentration across all treatment conditions.

Working solutions for in vivo studies: For intraperitoneal administration, prepare formulation vehicles by sequential addition: dissolve Withaferin-A in DMSO (typically 5 to 10 percent of final volume), add PEG300 and mix until clear, add Tween 80 (1 to 2 percent) and mix, then add saline or phosphate-buffered saline to final volume. Alternative vehicles include DMSO/corn oil and DMSO/cremophor EL/saline. Prepare fresh daily or within 24 hours of administration.

Handling precautions: Withaferin-A is a biologically active compound with demonstrated cytotoxic, pro-apoptotic, and anti-inflammatory activity. Handle with appropriate personal protective equipment (gloves, laboratory coat, eye protection). Avoid inhalation of powder. The electrophilic pharmacophores that confer biological activity also confer potential skin-sensitizing and irritant properties. Dispose of waste solutions according to institutional hazardous-chemical waste protocols.

Stability considerations: The alpha,beta-unsaturated ketone and epoxide pharmacophores are susceptible to nucleophilic degradation in aqueous solution, particularly at elevated pH. Aqueous working solutions should be prepared fresh and used within hours. Degradation is accelerated by light and elevated temperature. The compound is stable as a dry solid under recommended storage conditions for at least 3 years.

10. Stack Interactions and Combinations

Withaferin-A interacts with several compound classes through its multi-target pharmacology and through pharmacokinetic mechanisms. Investigators conducting research with Withaferin-A, especially in combination protocols, should consider:

Proteasome inhibitors: MG132, bortezomib, and other proteasome inhibitors reverse the Hsp90 client protein degradation induced by Withaferin-A [8]. Concurrent use antagonizes this specific mechanism while preserving the NF-kappaB and vimentin targeting. The combination may be useful for mechanism dissection but is unlikely to be therapeutically productive.

Conventional chemotherapy: Preclinical combination studies with doxorubicin, cisplatin, paclitaxel, and 5-fluorouracil have demonstrated additive or synergistic cytotoxicity in multiple cancer cell lines. The NF-kappaB inhibition by Withaferin-A may sensitize cancer cells to chemotherapy-induced apoptosis by suppressing survival signaling. The combination with liposomal doxorubicin is under clinical investigation in ovarian cancer.

Other NF-kappaB inhibitors: Concurrent use with other NF-kappaB pathway inhibitors (curcumin, celastrol, BAY 11-7082, TPCA-1) produces additive or redundant pathway inhibition. The combination has been explored in preclinical studies for enhanced anti-inflammatory effects but may increase the risk of immunosuppression.

CYP3A4 inhibitors and inducers: Although the specific CYP isoforms responsible for Withaferin-A metabolism have not been definitively identified, the rapid microsomal degradation suggests involvement of high-capacity CYP enzymes. Strong CYP3A4 inhibitors (ketoconazole, itraconazole, ritonavir, clarithromycin) may increase Withaferin-A systemic exposure, while strong CYP3A4 inducers (rifampin, carbamazepine, phenytoin, St. John's wort) may decrease exposure. These interactions have not been formally characterized but should be considered in research protocols.

Hsp90 N-terminal inhibitors: Concurrent use with geldanamycin-class or resorcinol-class Hsp90 inhibitors (17-AAG, ganetespib, luminespib) that bind the N-terminal ATP pocket produces dual-site Hsp90 inhibition. The combination has been explored in preclinical models for enhanced client protein degradation and may overcome the compensatory heat shock factor 1 activation that limits N-terminal inhibitors alone.

Leptin and metabolic agents: In the context of metabolic research, Withaferin-A has been combined with exogenous leptin to demonstrate synergistic effects on food intake reduction and weight loss in diet-induced obese mice [13]. Concurrent use with metformin, GLP-1 receptor agonists, or other metabolic agents has not been formally studied.

Immunotherapy: The NF-kappaB inhibition by Withaferin-A may modulate immune responses relevant to checkpoint inhibitor therapy. Preclinical studies suggest that Withaferin-A can enhance antitumor immunity in some contexts while suppressing it in others. The interaction with anti-PD-1, anti-PD-L1, and anti-CTLA-4 immunotherapy has not been formally characterized and represents an area of active investigation.

11. Adverse Events and Safety

11.1 Preclinical toxicology

The LD50 of Withaferin-A following oral administration in mice exceeds 2000 mg/kg body weight, classifying the compound as GHS category 5 (low acute toxicity) [16]. Patel et al. (2022) conducted a comprehensive 28-day oral toxicity study in mice at doses up to 500 mg/kg and reported no features of drug-induced toxicity on physiological, serum chemistry, hematological, or histopathological examination, establishing the No-Observed Adverse Effect Level (NOAEL) at 500 mg/kg or greater [16].

Subacute toxicity studies of *Withania somnifera* extracts standardized for Withaferin-A content have similarly reported favorable safety profiles, with no-observed-adverse-effect levels at multiples of projected human therapeutic doses [30].

At pharmacologically active doses in cancer xenograft studies (2 to 8 mg/kg intraperitoneal in mice), Withaferin-A is generally well tolerated without significant body weight loss or organ toxicity. Some studies have reported transient weight loss at higher doses (greater than 10 mg/kg), particularly with daily dosing.

11.2 Clinical adverse events

In the Phase I osteosarcoma trial (Pires et al. 2019), 11 adverse events of grade 1 or grade 2 severity were observed in 8 of 13 patients at oral doses of 72 to 216 mg per day [17]. The adverse event profile was:

- Liver enzyme elevation (5 events; the most common adverse event, suggesting hepatic stress consistent with the extensive hepatic metabolism of the compound)
- Skin rash (2 events)
- Fatigue (1 event)
- Fever (1 event)
- Edema (1 event)
- Diarrhea (1 event)

No grade 3 or grade 4 adverse events were observed. No dose-limiting toxicity was identified. The maximum tolerated dose was not reached at the highest administered dose (216 mg per day).

11.3 Hepatic considerations

The liver enzyme elevation observed in the Phase I trial is consistent with the extensive hepatic metabolism of Withaferin-A and with the covalent pharmacology (reactive electrophiles can form adducts with hepatocyte proteins). Investigators should monitor liver function tests in chronic dosing protocols and should consider the hepatic safety signal as a potential dose-limiting factor in clinical development.

11.4 Reproductive and developmental toxicity

Formal reproductive and developmental toxicity studies of purified Withaferin-A have not been published. Withania somnifera extract studies in rodents have not demonstrated teratogenicity at studied doses. However, the covalent multi-target pharmacology and the cytotoxic activity of Withaferin-A warrant caution. Use in pregnancy, lactation, and in women of childbearing potential without contraception should be avoided in research protocols.

11.5 Immunosuppressive potential

The NF-kappaB inhibition and anti-inflammatory activity of Withaferin-A have immunosuppressive implications. Chronic dosing at anti-inflammatory concentrations could theoretically impair innate immune responses. This concern is theoretical at clinical doses and has not been observed in the Phase I trial or in ashwagandha extract clinical studies, but should be monitored in longer-duration protocols.

12. Comparable Compounds Assessment

This section presents a structured comparative assessment of five natural-product anticancer and anti-inflammatory candidates against Withaferin-A, evaluated against five competency standards: novelty, effect size, promising potential (current clinical state and trajectory), side-effect profile, and overall validation. The five candidates were selected as the principal plant-derived compounds with established NF-kappaB inhibitory, anticancer, or multi-target pharmacology that occupy the same translational niche as Withaferin-A, with priority given to compounds that have produced material clinical evidence.

12.1 Celastrol

Mechanism: Celastrol is a pentacyclic triterpenoid isolated from *Tripterygium wilfordii* (thunder god vine) that inhibits NF-kappaB signaling, Hsp90, and proteasome function through covalent modification of reactive cysteine residues, a mechanism broadly analogous to Withaferin-A [31]. The compound also functions as a leptin sensitizer in diet-induced obese mice, a property shared with Withaferin-A.

Novelty: Low to moderate. The NF-kappaB and Hsp90 inhibitory mechanisms are shared with Withaferin-A and with other covalent natural-product electrophiles. The leptin-sensitizing activity was reported by Liu et al. (2015, *Cell*) and generated substantial interest [32].

Effect size: Preclinical anticancer efficacy is comparable to Withaferin-A in magnitude (50 to 70 percent tumor growth inhibition in xenograft models at studied doses). Anti-obesity effect sizes in mice are substantial (greater than 40 percent weight reduction). No clinical efficacy data are available for purified celastrol.

Promising potential: Low. Clinical development is substantially limited by hepatotoxicity and narrow therapeutic index. No clinical trial of purified celastrol for oncology or metabolic indications has been completed. The compound remains a preclinical research tool.

Side-effect profile: Hepatotoxicity is the principal concern. Celastrol produces dose-dependent liver injury in rodents at doses close to the pharmacologically active range, representing a narrow therapeutic index. Additional toxicities include infertility in male rodents (reversible) and gastrointestinal irritation.

Overall validation: Low to moderate. Extensive preclinical data with strong mechanistic characterization, but clinical translation is blocked by hepatotoxicity.

Comparison to Withaferin-A: Celastrol shares the covalent multi-target pharmacology and the leptin-sensitizing property with Withaferin-A, but has a more concerning hepatotoxicity profile. Withaferin-A has the advantage of a completed Phase I clinical trial demonstrating tolerability, a wider therapeutic index in preclinical models, and a more favorable safety record from the ashwagandha extract clinical literature.

12.2 Curcumin

Mechanism: Curcumin is a polyphenolic diarylheptanoid isolated from *Curcuma longa* (turmeric) that inhibits NF-kappaB, STAT3, AP-1, and multiple kinases through a combination of covalent Michael addition chemistry (at the alpha,beta-unsaturated carbonyl groups) and non-covalent binding [33]. The compound possesses anticancer, anti-inflammatory, and antioxidant properties.

Novelty: Low. Curcumin is the most extensively studied natural-product NF-kappaB inhibitor with a research literature spanning more than 10,000 publications. The pharmacology is well established but not novel.

Effect size: Modest in clinical trials. Despite extensive preclinical efficacy data, clinical trials of curcumin in cancer and inflammatory conditions have generally shown small effect sizes that have not supported registration. A principal limitation is the extremely low oral bioavailability (less than 1 percent for standard curcumin) and rapid metabolism [34].

Promising potential: Low to moderate. Decades of research have not produced a registered therapeutic application for purified curcumin. Enhanced bioavailability formulations (piperine co-administration, liposomal curcumin, nanoparticle curcumin) have improved pharmacokinetics but have not yet produced breakthrough clinical efficacy.

Side-effect profile: Favorable. Curcumin is well tolerated at oral doses up to 12 grams per day in clinical studies, with gastrointestinal adverse events (nausea, diarrhea) as the principal dose-limiting effects [34].

Overall validation: Moderate. The compound has the largest clinical research literature of any natural-product anticancer candidate, but clinical effect sizes have been consistently disappointing relative to preclinical promise.

Comparison to Withaferin-A: Curcumin shares the NF-kappaB inhibitory mechanism and the bioavailability limitation with Withaferin-A, but has a substantially more extensive clinical trial record (albeit with modest results). Withaferin-A has higher potency (IC50 values typically 10- to 100-fold lower), more defined covalent target engagement (vimentin, Hsp90), and additional mechanisms (leptin sensitization, Hsp90 C-terminal binding) that curcumin does not possess. The curcumin clinical experience provides a cautionary precedent for Withaferin-A translational expectations.

12.3 Sulforaphane

Mechanism: Sulforaphane is an isothiocyanate derived from glucoraphanin in cruciferous vegetables (principally broccoli sprouts) that activates the Nrf2 antioxidant response pathway through covalent modification of KEAP1 cysteine residues [35]. The compound also inhibits histone deacetylases (HDACs), NF-kappaB, and cancer stem cell self-renewal pathways.

Novelty: Low to moderate. The Nrf2/KEAP1 mechanism is shared with other isothiocyanates and with Withaferin-A (which also modifies KEAP1). The HDAC inhibitory activity adds a dimension not present in Withaferin-A.

Effect size: Modest to moderate in cancer prevention clinical trials. Sulforaphane has demonstrated modulation of Phase II detoxification enzymes and reduction of aflatoxin-DNA adducts in human studies [36]. Anticancer efficacy in therapeutic (rather than preventive) contexts has not been established clinically.

Promising potential: Moderate. The cancer chemoprevention application is the most clinically advanced, with multiple Phase 2 trials in prostate cancer, breast cancer, and bladder cancer reporting biomarker modulation. Registration trials have not been completed.

Side-effect profile: Favorable. Well tolerated at dietary and supplemental doses. Gastrointestinal effects (flatulence, bloating) are the principal adverse events at higher doses.

Overall validation: Moderate. Stronger clinical evidence base for cancer chemoprevention than for cancer therapy. The dietary-supplement regulatory pathway provides an alternative to pharmaceutical registration.

Comparison to Withaferin-A: Sulforaphane shares the KEAP1/Nrf2 mechanism with Withaferin-A but lacks the vimentin, Hsp90, and NF-kappaB inhibitory activities. Sulforaphane is better positioned for cancer chemoprevention applications, while Withaferin-A is better positioned for cancer therapy applications on the basis of its broader target engagement and higher potency.

12.4 Parthenolide

Mechanism: Parthenolide is a sesquiterpene lactone isolated from *Tanacetum parthenium* (feverfew) that inhibits NF-kappaB through direct alkylation of the p65 subunit at Cys38 and through inhibition of IKKbeta [37]. The compound also targets cancer stem cells through oxidative stress induction and HDAC1 inhibition.

Novelty: Moderate. The direct p65 alkylation mechanism is distinct from the IKKbeta-targeting mechanism of Withaferin-A, and the cancer stem cell selectivity has generated considerable research interest.

Effect size: Substantial in preclinical leukemia models. Parthenolide selectively targets leukemia stem cells while sparing normal hematopoietic stem cells in vitro [37]. In vivo xenograft efficacy has been demonstrated across multiple tumor types. No clinical trial of purified parthenolide has been completed; the water-soluble prodrug dimethylamino-parthenolide (DMAPT) was advanced to Phase I for acute myeloid leukemia but results have not been published.

Promising potential: Low to moderate. The prodrug DMAPT addressed the bioavailability limitation but the clinical trajectory has not progressed beyond Phase I.

Side-effect profile: Preclinical only for purified compound. Feverfew extracts containing parthenolide have a long history of use for migraine prophylaxis with a favorable safety record.

Overall validation: Low to moderate. Strong preclinical data, particularly for leukemia stem cells, but clinical validation is absent.

Comparison to Withaferin-A: Parthenolide shares the NF-kappaB inhibitory mechanism but targets p65 directly rather than IKKbeta. Withaferin-A has broader target engagement, a completed Phase I clinical trial, and established preclinical efficacy across more cancer types. Parthenolide has the advantage of cancer stem cell selectivity in leukemia models.

12.5 Gambogic acid

Mechanism: Gambogic acid is a xanthone derived from *Garcinia hanburyi* (gamboge) resin that induces apoptosis through multiple mechanisms including Bcl-2 family modulation, topoisomerase II inhibition, transferrin receptor targeting, and NF-kappaB suppression [38]. The compound has been studied most extensively in Chinese preclinical and early clinical oncology programs.

Novelty: Low to moderate. The transferrin receptor targeting provides tumor selectivity (cancer cells overexpress the transferrin receptor), a dimension not present in Withaferin-A.

Effect size: Moderate to substantial in preclinical models, with IC50 values in the submicromolar to low-micromolar range across multiple cancer cell lines. Phase II clinical trials in China have reported partial responses in non-small cell lung cancer and other solid tumors, though the data quality and reporting standards of some studies have been questioned in Western oncology literature [38].

Promising potential: Low to moderate outside China. The compound has not entered clinical trials in Western regulatory jurisdictions. Chinese clinical data report activity but have not led to international registration.

Side-effect profile: Dose-dependent hepatotoxicity and hematological toxicity have been reported in clinical studies. The therapeutic index is narrower than that of Withaferin-A.

Overall validation: Moderate in Chinese clinical contexts; low in international regulatory contexts.

Comparison to Withaferin-A: Gambogic acid has more advanced clinical development (Phase II in China) but a narrower therapeutic index and greater hepatotoxicity. Withaferin-A has a more favorable safety profile, broader multi-target pharmacology, and additional metabolic and neuroprotective applications that gambogic acid does not possess.

12.6 Comparator summary

The five compounds occupy distinct positions in the natural-product anticancer pharmacology space. **Celastrol** shares the closest mechanistic similarity to Withaferin-A (covalent multi-target, NF-kappaB/Hsp90 inhibition, leptin sensitization) but is limited by hepatotoxicity and has not reached clinical evaluation. **Curcumin** has the most extensive clinical research literature but has consistently disappointed in therapeutic efficacy, providing a cautionary precedent for natural-product anticancer translation. **Sulforaphane** is best positioned for cancer chemoprevention rather than therapy and has a more limited target profile. **Parthenolide** has compelling leukemia stem cell selectivity but has not progressed beyond early clinical investigation. **Gambogic acid** has the most advanced clinical development (Phase II in China) but with hepatotoxicity concerns and limited international validation.

Withaferin-A occupies a distinctive position in this landscape as the only compound with: (a) a completed Phase I clinical trial demonstrating tolerability at pharmacologically relevant doses; (b) the broadest validated multi-target covalent pharmacology (vimentin, IKKbeta, Hsp90, annexin II, beta-tubulin, KEAP1, BTK); (c) established preclinical efficacy across the greatest number of cancer types; and (d) additional metabolic (leptin sensitization, adipose browning) and neuroprotective applications not present in the comparators. The principal limitation shared with curcumin and celastrol is low oral bioavailability, which represents the critical translational barrier.

The comparative assessment indicates that Withaferin-A is the most pharmacologically versatile and the most clinically advanced (by Phase I completion) of the natural-product multi-target anticancer candidates evaluated. The translational path forward depends on resolution of the bioavailability limitation through advanced formulation strategies and on the design of clinical trials with appropriate bioanalytical methods for pharmacokinetic characterization.

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