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Development of withaferin A analogs as probes of angiogenesis

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Abstract—The natural product withaferin A (WFA) is a potent angiogenesis inhibitor and it targets the ubiquitin–proteasome pathway in vascular endothelial cells. We generated a biotinylated affinity analog WFA-LC_2B for use as a probe to study angiogenesis. WFA-LC_2B inhibits angiogenic sprouting in vitro and it causes levels of ubiquitinated proteins to increase in tumor necrosis factor-α-treated human umbilical vein endothelial cells, confirming the retention of WFA's biological activity. We show that WFA-LC_2B forms protein adducts in endothelial cells which are competed by free WFA in vivo. This WFA-LC_2B analog will be useful to isolate the biological target of WFA.

Withaferin A (WFA), an important prototype of the withanolide class of natural products (Fig. 1), is a highly oxygenated steroidal lactone that is found in the medicinal plant *Withania somnifera* and its related solanaceas species.[1] The withanolides are known to exert very potent and diverse cytotoxic, anti-stress, cardioactive, central nervous system, and immunomodulatory activities.[2] Since the early discovery of WFA during the 1960s, the major interest has been on its anti-tumor cytotoxic activities.[3,4] However, the non-cytotoxic anti-inflammatory[5] and immunomodulatory mechanisms[6] of WFA have thus far remained rather poorly characterized. These latter disease-altering activities are highly pertinent to the practice of ayurveda, a traditional form of Indian medicine, which has borne out many effective formulations from *W. somnifera*, especially for the treatment of chronic human diseases such as arthritis and female bleeding disorders.[2]

Angiogenesis, which is the growth of new blood vessels from preexisting vasculature, is a pathogenic manifestation in cancers,[7] and it is also widely recognized to be critically involved in the pathogenesis of arthritis, endometriosis, age-related macular degeneration, diabetic retinopathy, etc.[7] Since these non-malignant inflammatory diseases could also benefit from anti-angiogenic therapeutics,[8] new treatments for such chronic diseases have spurred a greater interest for angiogenesis inhibitors.

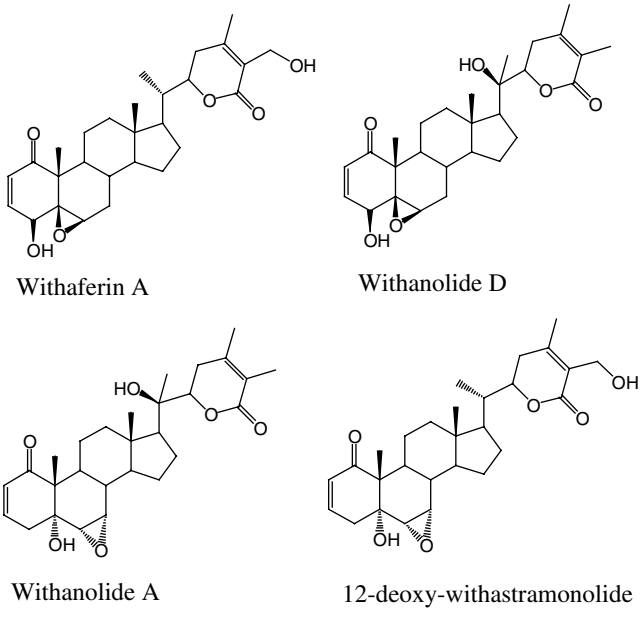

Figure 1. Some of the major withanolides found in *Withania somnifera*.

Keywords: Biotinylated analog; Natural product; Binding protein; Ubiquitin; Angiogenesis inhibitor.
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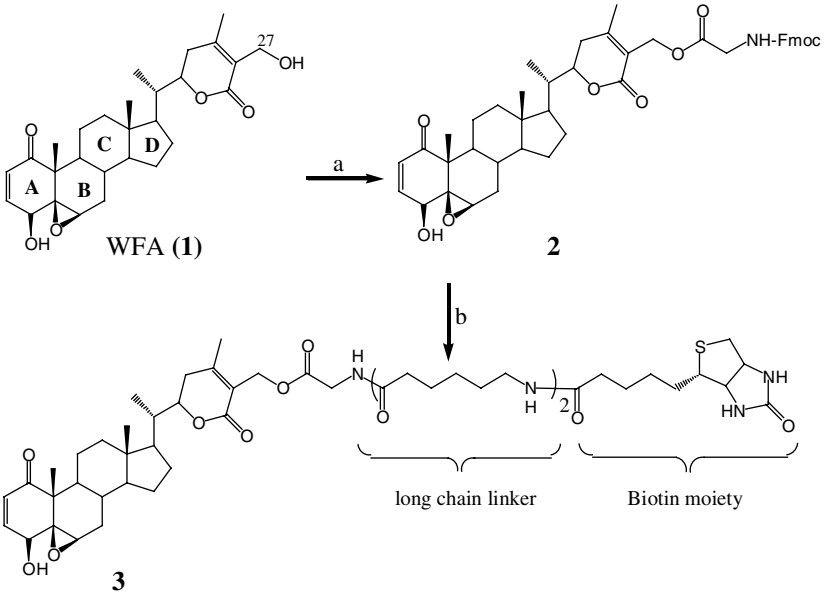
We have been intrigued by the Ayurvedic use of *W. somnifera*-based formulations in the treatment of arthritis and menstrual disorders in women.[9] And therefore, we hypothesized that such extracts could possess heretofore unrecognized inhibitors of angiogenesis. In fact, we demonstrated that *W. somnifera* extracts containing non-cytotoxic levels of withanolides, and also WFA, the derived active principle of these extracts, exert potent anti-angiogenic activity in vivo at very low doses.[10] Furthermore, at low nanomolar concentrations, we showed that WFA directly targets endothelial cell proliferation and exerts cytostatic cell cycle G_1 arrest in human umbilical vein endothelial cells (HUVECs). Interestingly, non-cytotoxic sub-to-low micromolar concentrations of WFA also inhibit in vitro vessel formation[10,11] in the three-dimensional endothelial cell sprouting assay (3D-ECSA). At such doses, WFA potently inhibits TNF-α-induced NF-κB-DNA-binding activity, a mechanism which is associated with stabilization of phosphorylated IκB-α in the cytoplasm. Our findings suggest that WFA does not interfere with upstream phosphorylation events, but acts possibly at the level of protein ubiquitination/degradation. In fact, we showed that WFA increases global levels of ubiquitinated-protein species in TNF-α-treated HUVECs, suggesting that the ubiquitin–proteasome pathway (UPP) is the major target of this class of natural product.[10] Given the critical role that ubiquitination plays in cell cycle control, differentiation, and NF-κB signaling,[12] it is readily recognized that an important key step to study the anti-angiogenic mode of action of WFA is to identify its cellular biological target(s).

In this study, we sought to detect the direct binding protein(s) of WFA. Fortunately, previous structure–activity studies have identified the relevant pharmacophores of WFA, which indicate the importance of the 4β-hydroxy-5β,6β-epoxy-2-en-1-one moiety and unsaturated lactone side chain (Fig. 1) for biological activity.[13,14]

Notably, the unsaturated A-ring is known to react with thiol-nucleophiles and undergoes Michael addition, and the unsaturated lactone is readily alkylated by model biological nucleophiles that mimic proteins.[15] In fact, we recently showed the requirement for the A-ring unsaturation for sprouting inhibitory activity in the 3D-ECSA, because conversion of WFA to 3-methoxy-2,3-dihydrowithaferin A abrogates this activity.[11] However, the C27 hydroxyl in WFA is known to be dispensable for biological activity;[15] this fortuitous functional group was therefore exploited to attach a biotin adduct through a 12-hydrocarbon chain linker to afford the generation of biotinylated analog of WFA (Scheme 1). This chemical genetic strategy[16] was chosen because we and others have employed such long-hydrocarbon chain linkers to couple natural products to biotin and successfully employed these affinity reagents to identify binding targets of diverse natural products, such as fumagillin, epoxomicin, parthenolide, etc.[17] Therefore, following these established synthetic chemical procedures, WFA (**1**) was first coupled with Fmoc-Gly-OH to generate the intermediate Fmoc-Gly-WFA (**2**), which was purified by silica gel flash chromatography.[18] The subsequent deprotection of Fmoc group and reaction with NHS-LC-LC-biotin afforded WFA-Gly-LC-LC-biotin **3** (WFA-LC_2B). WFA-LC_2B was purified by silica gel flash chromatography and structure validated using mass spectroscopy and ^{1}H NMR spectroscopy.[19]

To ensure the preservation of biological activity of WFA-LC_2B, we first tested this affinity reagent in the 3D-ECSA, an assay we have established to be highly sensitive to inhibitors of the UPP.[10,11,20] Spheroids derived from HUVECs were embedded in a collagen I



Scheme 1. Synthesis of WFA-LC_2B. Reagents and conditions: (a) i—Fmoc-Gly-OH, oxalyl chloride/DMF, CH_2Cl_2, rt (3 h); ii—DMAP, CH_2Cl_2, rt (3 h); (b) i—20% piperidine/DMF (15 min); ii—NHS-LC-LC-biotin, DMSO, rt (30 min).

matrix and provided with 20 ng/ml bFGF to induce sprouting.

The effect of WFA, 12-deoxy withastramonolide (12-D WS), and WFA-LC_2B on angiogenic sprouting was investigated by co-treatment of these spheroids for 20 h. As anticipated, 1 μM WFA potently blocked bFGF-induced vessel growth in the collagen I matrix, whereas its regioisomeric congener, 12-D WS (Fig. 1), even at 5 μM did not inhibit angiogenic sprouting (Fig. 2). As evidenced from spheroid images, WFA-LC_2B completely inhibits sprouting at 5 μM, whereas at 2 μM only a few sprouts are formed and their growth is also attenuated considerably (Figs. 2A and B). At concentrations below 500 nM WFA-LC_2B we did not observe any inhibition of sprouting (data not shown). We estimated from dose–response studies in the 3D-ECSA that there is a 5- to 8-fold loss in potency with WFA-LC_2B compared to WFA (data not shown), which results possibly from reduced cell permeability. Such loss in potency has previously been reported with biotinylated natural products although this permeability limitation has not restricted their successful use for target identification.[17,21]

Next, to investigate whether WFA-LC_2B retains the UPP-targeting activity of WFA, we tested WFA-LC_2B in HUVEC cultures and analyzed protein ubiquitination by Western-blot analysis as done previously.[10] HUVECs pre-treated with 5 μM WFA-LC_2B for 1 h and subsequently with TNF-α for 20 min were shown to abundantly accumulate high molecular weight ubiquitinated-protein species compared to TNF-α-alone-treated cells (Fig. 3). Similar treatment at the lower dose of 1 μM WFA-LC_2B produced a weaker response.

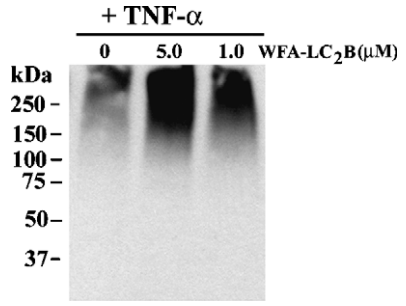


Figure 3. HUVECs were treated for 1 h with DMSO (0) or with different doses of WFA-LC_2B. The treated cells were subsequently stimulated with TNF-α (10 ng/ml) for 20 min and then harvested. Equal amounts of protein from cytoplasmic extracts were subjected to SDS–PAGE on 4–20% gradient gels and Western blotted using a monoclonal antibody against ubiquitin. Antibody complexes were detected with a goat anti-mouse-horseradish peroxidase (HRP) conjugate and visualized with chemiluminescence detection.

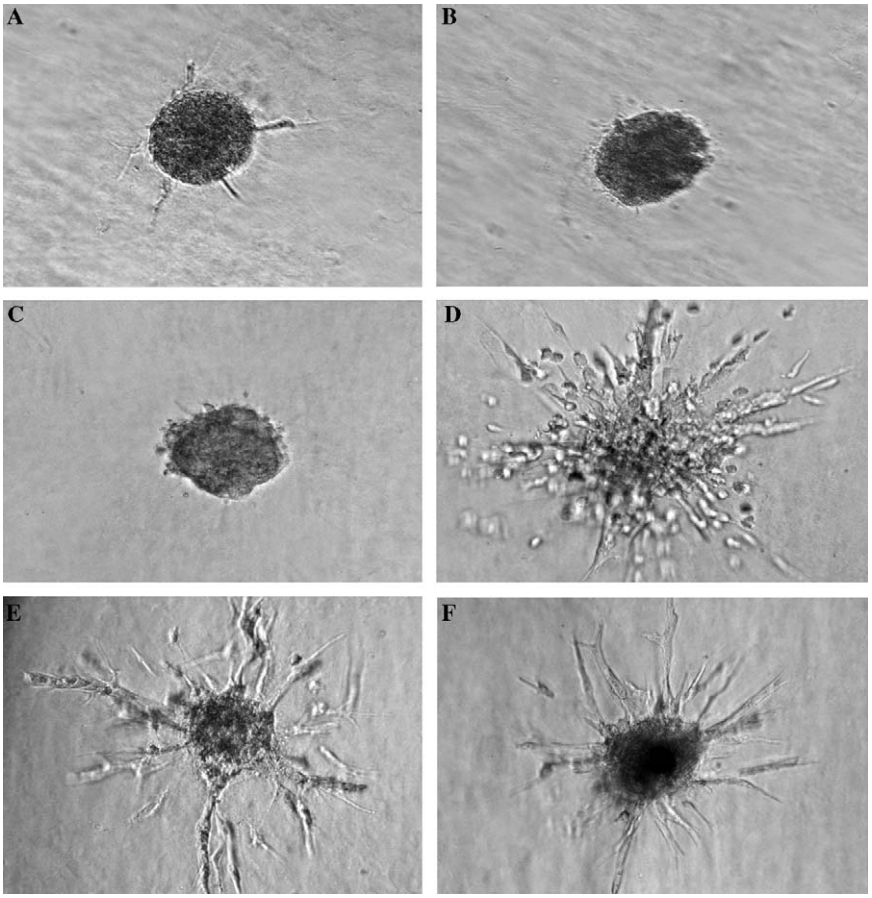


Figure 2. Endothelial cell spheroids in 96-well plates were incubated with growth factor in the presence and absence of WFA analogs for 20 h. Digital photographs of samples were obtained on an inverted microscope at 10× magnification. Representative images are shown of spheroids treated with 20 ng/ml bFGF along with 2 μM WFA-LC_2B (A), 5 μM WFA-LC_2B (B), 5 μM WFA (C), 5 μM 12-D WS (D), control samples treated with 20 ng/ml bFGF (E) or VEGF (F) alone.

This pattern of ubiquitination is consistent with mode of action of WFA in HUVECs.[10] Thus, our findings show that WFA-LC_2B is biologically active in HUVECs and its activities parallel that of WFA.[10]

Finally, to detect the binding protein targets of WFA, we investigated the ability of WFA-LC_2B to form adducts with cellular proteins in vivo.[22] HUVECs were pre-treated with DMSO or 5 μM WFA for 30 min and subsequently incubated with 5 μM WFA-LC_2B for 4 h. Alternatively, in reverse competition experiments HUVECs were pre-treated with 5 μM WFA-LC_2B for 1 h and subsequently with DMSO or 5 μM WFA for 4 h. Cytoplasmic protein samples were subjected to affinity chromatography on Ultra-link immobilized NeutrAvidin™ beads and the bound proteins eluted.

SDS–PAGE of affinity-purified proteins with silver staining analysis identified ~180, 70, and 56 kDa protein species that are bound by the WFA-LC_2B affinity reagent and competed by free WFA in vivo (Fig. 4A). The reverse competition experiment revealed a partial reduction in the intensity of only the 70 kDa band (Fig. 4B). These findings suggest that WFA binds presumably via irreversible covalent interactions with the abundant 56 kDa protein target and the less abundant 180 kDa species, whereas its interactions with the 70 kDa protein species appear to be reversible.

In conclusion, we have generated a biologically active affinity-tagged analog of WFA, WFA-LC_2B. This versatile small molecule probe will allow us to isolate the protein targets of WFA by affinity chromatography, studies which are currently underway in our laboratory. The identification of the biological targets of WFA should prove useful in the study of the UPP and angioinflammatory signal transduction.

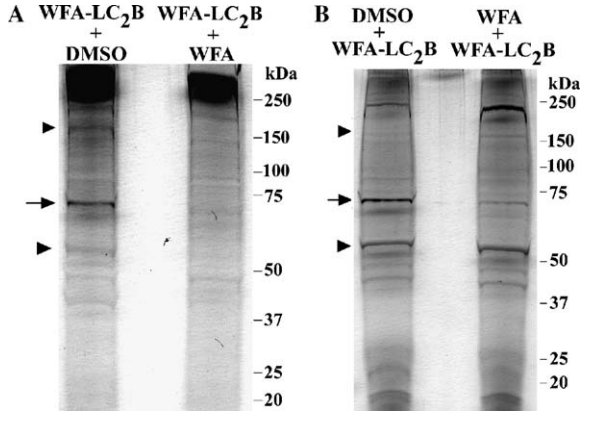


Figure 4. HUVECs were treated first with DMSO or 5 μM WFA for 30 min and subsequently with 5 μM WFA-LC_2B for 4 h (A), or alternatively first with 5 μM WFA-LC_2B for 1 h and subsequently with DMSO or 5 μM WFA for 4 h (B). Cytoplasmic extracts were precleared and loaded on separate NeutrAvidin columns. The columns were washed and bound biotinylated proteins eluted and subjected to SDS–PAGE on 4–20% gradient gels. Silver staining revealed that one major (arrow) and at least two minor (arrowheads) biotinylated proteins were competed by free WFA in (A), but only the 70 kDa band was competed in (B).

Acknowledgments

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18. Compound **2**: Fmoc-Gly-OH (15.5 mg, 0.052 mmol) was dissolved in 1.5 ml CH_2Cl_2, added with 0.13 ml of oxalyl chloride (2 M in CH_2Cl_2, 0.13 mmol) and a drop of DMF at room temperature. The mixture was stirred for 15 min. The solvent was removed under vacuum for 2 h and the white-yellow paste was dissolved in 5.0 ml CH_2Cl_2, then 0.5 ml of the solution was added to a mixture of WFA (6.8 mg, 0.013 mmol, Chromadex), and DMAP (2.08 mg, 0.017 mmol) in 2 ml of CH_2Cl_2 at 0 °C and stirred for 3 h. The reaction mixture was poured into water, extracted with CH_2Cl_2, washed with sat. Na_2CO_3, then with brine, dried over Na_2SO_4, concentrated, and purified by silica gel

flash chromatography (CH_2Cl_2/MeOH 100:0 to 98:2) to yield **2** (2.3 mg, 60% yield calculated based on Fmoc-Gly-OH). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (d, J = 7.2 Hz, 2H), 7.61 (d, J = 6.9 Hz, 2H), 7.42 (t, J= 7.5 Hz), 7.34 (dd, J = 7.2, 1.2 Hz, 2H), 6.94 (dd, J = 10.0, 5.7 Hz, 1H), 6.21 (d, J = 10.0 Hz, 1H), 5.30 (m, 1H), 5.00 (br s, 2H), 4.41 (m, 2H), 4.24 (m, 1H), 4.01 (d, J = 5.7 Hz, 2H), 3.78 (d, J = 5.7 Hz, 1H), 3.25 (br s, 1H), 2.50 (m, 1H), 2.2–0.8 (m, 30H); MS (MALDI): m/z = 788 (M+K), 772 (M+Na), calcd for $C_{45}H_{51}NO_9$: m/z = 749.36.

19. Compound **3**: The intermediate **2** (12.0 mg, 0.016 mmol) was added with 20% piperidine/DMF (0.5 ml) at room temperature and stirred for 15 min. The volatiles were removed under vacuum for 5 h. The crude free amine was dissolved in DMSO (1.5 ml), Biotin-$(LC)_2$-NHS (Pierce; 13.6 mg, 0.024 mmol) was added and stirred at room temperature for 30 min. The solvent was removed under vacuum overnight. The crude product was purified by silica gel flash chromatography (EtOAc/MeOH 95:5 to 80:20) to yield **3** (7.1 mg, 45%). ^{1}H NMR (300 MHz, $CDCl_3$/ CD_3OD): δ 6.99 (dd, J = 8.4, 6.0 Hz, 1H), 6.61 (m, 1H), 6.30 (d, J = 10 Hz, 1H), 6.07 (m, 1H), 5.53 (m, 1H), 5.30 (s, 1H), 4.80 (d, J = 6.0 Hz, 1H), 4.50 (m, 1H), 4.33 (m, 1H), 3.3–3.1 (m), 2.95 (dd, 1H), 2.9–2.7 (m), 2.4–2.0 (m), 1.8–1.2 (m); MS (ESI):980 (M^+), calcd for $C_{52}H_{77}N_5O_{11}S$: m/z = 979.53.
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22. Three 100 mm dishes of HUVECs were employed for each treatment. The treated cells were lysed by multiple passage through tuberculin syringe needle at 4 °C in 25 mM Hepes, pH 7.6, 1% Triton X-100, 150 mM NaCl, and 50 mM NaF with the inclusion of 1 μM PMSF and proteinase inhibitor cocktail (Roche). Proteins were quantified using the Bio-Rad reagent and equal amounts of protein pre-cleared with Sepharose beads (0.2 ml, 30 min) and then loaded repeatedly on Ultra-link immobilized NeutrAvidin beads (0.2 ml, Pierce). The bead columns were washed five times with 0.5 ml of ice-cold lysis buffer, and bound proteins eluted by boiling the beads in SDS–PAGE sample loading buffer.