PLEIOTROPIC EFFECTS OF WHITE WILLOW BARK AND 1,2 DECANEDIOL ON HUMAN ADULT KERATINOCYTES

SHORT COMMUNICATION

PLEIOTROPIC EFFECTS OF WHITE WILLOW BARK AND 1,2 DECANEDIOL ON HUMAN ADULT KERATINOCYTES.

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Running title: Effects of white willow bark and 1,2 decanediol on keratinocytes.
Keywords: natural products, keratinocytes, 1,2 decanediol, white willow bark

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Abstract:

BACKGROUND: *Acne vulgaris* is a common skin defect, usually occurring during adolescence, but often it can persist in adults leaving permanent face scarring. *Acne* is usually treated with topic drugs, oral antibiotics, retinoids and hormonal therapies, but medicinal plants are increasingly employed.

OBJECTIVE: To investigate the protective role of white willow bark (WWB) and 1,2 decanediol (DD) on the damage caused by Lipopolysaccharides (LPS) on human adult keratinocytes (HaCaT).

METHODS: HaCaT were exposed to LPS alone or in association with WWB and DD. Epidermal viability, metabolic modulation, inflammatory activity, and cell migration were assessed with both common standardized protocols or high-throughput screening systems (HTS).

RESULTS: The pre-incubation of HaCaT with WWB and DD (used separately or in combination) differently prevented the alterations induced by LPS on HaCaT in terms of growth factor release (IGF, EGF, VEGF), cytokine production (IL-1α, IL-6, IL-8), or expression of the transcription factor FOXO-I. Moreover, they partially restore wound repair lowered by LPS.

CONCLUSIONS: These results suggest that both natural compounds were able to differently affect several functions of LPS-stressed keratinocytes suggesting their potential role for prevention of acne vulgaris, without adverse effects.

INTRODUCTION

Human skin is a complex organ which plays many functions such as regulation of water/electrolyte homeostasis, defense against physical, chemical and biological factors and secretion [1-2]. Skin is formed by different cell types in a well-structured interplay among epidermal and follicular keratinocytes, sebocytes, melanocytes, dermal papilla cells, fibroblasts, endothelial cells and sweat gland cells [3]. It is a target for hormones and an endocrine gland, whose physiology is influenced by environmental, genetic or nutritional factors. The primary mechanism of skin cell alteration is based on oxidative stress processes [4].

Acne vulgaris is a very common defect, tipically associated with adolescence, but sometimes leading to permanent scarring on the face in adults. Its complex development involves androgen-mediated stimulation of sebaceous gland activity, follicular hyper-keratinization and colonization of Propionibacterium acnes (P. Acnes). *Acne* is currently treated by the use of local applications, oral antibiotics, retinoids and hormonal therapies: nonetheless, increasing interest is focused on natural plant-derivatives [5]. Some natural compounds (eg. mixtures of phenolic compounds) are recently under investigation as promising drugs for new dermal cosmetics that possess the ability to maintain skin cell renewal elastin and collagen stimulation) [6]. Mixed formulations (kaempferol and either erythromycin or clindamycin; quercetin and either erythromycin or clindamycin) synergically inhibit antibiotic resistant P. acnes growth [7]. The European Pharmacopoeia defines White Willow Bark (WWB) as the whole or fragmented dried bark of young branches or dried pieces of current year twigs from various species of the genus *Salix* [8]. WWB has been used as a traditional medicine for the treatment of fever, pain, and inflammation [9]. Salicin, the major constituent of WWB extract, is metabolized to salicylic acid *in vivo* and plays anti-inflammatory effects; moreover, other ingredients in the extracts include other salicylates as well as polyphenols and flavonoids, that could play prominent roles in the therapeutic efficacy [10]. WWB suppresses inflammatory molecules and reduces oxidative stress in human endothelial cells [11-12]. *In vitro*

and *in vivo* studies evidenced that the anti-inflammatory activity of WWB is associated with the downregulation of the inflammatory mediators TNF-α and NF-KB [13]. A work by Gopaul et al. (2010) described the ability of salicin to reduce the visible signs of skin aging when applied topically, thus showing anti-aging capabilities [14].

Among natural compounds involved in the regulation of skin homeostasis, 1,2 alkanediols display several properties (high water solubility and effectiveness as a solvent) that allow their use as moisturizing compounds in dermal cosmetic field [15-16]. Indeed, their bacteriostatic and fungistatic activity decreased the amount of conventional preservatives inside cosmetic formulations. Generally, the antimicrobial effect of 1,2-alkanediols increase with the length of their carbon chain, with 1,2-decanediol having a MIC value of <1%, which is 10-fold lower than that of 1,2-octanediol [17]. A recent study reported the effects of five 1,2-alkanediols on skin irritation potentials [18], that represent an important factor for determining the usefulness of 1,2-alkanediols.

Here we tested the effects of WWB and 1,2-decanediol (DD), used alone or in combination, on human adult keratinocytes (HaCaT) stressed by LPS. Moreover, we investigated a possible role of DD not only as a moisturizing agent, but also as bioactive molecule on epidermal layers.

Materials and methods

Drugs

Lipopolysaccharides from Escherichia coli (LPS) were purchased from Sigma-Aldrich. 1,2 decanediol (DD) is a diol monomer, solid and soluble only in cosmetic esters, and acts as an anti-acne agent and antiperspirant (Symrise, Holzminden, Germany). Willow bark extract (WWB) is an extract of the white bark of *Salix Alba* that is standardized for its salicin content (Euromed (Barcellona, Spain). DD and WWB were prepared in 0.001% DMSO (see table I for concentrations).

Cell Cultures

Human Adult Keratinocyte cell line (HaCaT) was obtained from Cell Line Services (CLS Cell Lines Service, Germany). HaCaT were grown in Dulbecco's Modified Eagle medium (DMEM) with 10% FCS and 1% antibiotic/antimycotic (Invitrogen, Grand Island, NY, USA).

Cell viability

HaCaT were seeded in 96-well plates (5000 cells/well). Cells were treated as indicated in Table II. After 24 hrs cell viability was evaluated by the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA), using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS). MTS conversion into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. Formazan product was measured with a FilterMax F5 Microplate reader (Molecular Devices, US) at 490 nm, as absorbance is directly proportional to the number of viable cells.

Enzyme-linked immunosorbent assay (ELISA)

Pro-inflammatory cytokines (IL-1α, IL-6 and IL-8), growth factors (VEGF, EGF, IGF-I), metabolic markers (keratin 16) and transcriptional factors (FOXO-I) were quantified in cell lysates or medium by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Sigma ELISA kit, Invitrogen ELISA Kit). Briefly, 100 µL of medium or cell lysate were incubated into an antibody-coated 96-well plate at room temperature for 2,5 hrs. The wells were washed four times with wash buffer solution. Then 100 µL of primary anti-human antibody was added and the samples were again incubated for 1 h at room temperature. The plate was washed four times, 100 µL of streptavidin-peroxidase conjugated was applied for 1 h at room temperature. After a final washing, 100 µL tetramethylbenzidine substrate was added and allowed to develop for 30' in the dark at room temperature. After stopping the reaction with 50 µL stop solution containing citric acid 2.0 $mmolL^{-}$

[1], absorbance was read at 450 nm with a F5 FilterMax microplate reader 550 (Molecular Devices, US). Sample concentration was calculated from the standard curve.

Cytosolic and nuclear protein extraction for FOXO-I quantification

HaCaT were grown to 80% confluence. Afterwards, cells were scraped using fresh PBS, collected into an appropriate conical tube and centrifuged (5' at 450 x *g*). Then the supernatant was discarded and 1 mL of Lysis Buffer (10 mM Tris HCl, pH 7.5, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.3 M Sucrose, including DTT and Protease Inhibitors) was added to 200 µL of packed cell volume (PCV) for 15 minutes. Suspended cells were centrifuged for 5 minutes at 420 x *g*. Pellet of packed cells was resuspended in 400 µL (2X PCV) Lysis Buffer and fragmented using a syringe with a narrow-gauge. The disrupted cells in suspension were centrifuged for 20 min at 10,000 x *g*. The supernatant was transferred into a fresh tube and this fraction corresponds to the cytoplasmic fraction. The pellet was resuspended in 140 µl extraction buffer (20 mM HEPES, pH 7.9, with 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) Glycerol added with 1.5 µL of the 0.1 M DTT solution and 1.5 µL of the Protease Inhibitor Cocktail) and centrifuged for 5 minutes at 20,000 x *g*. The resulting supernatant is the nuclear protein extract. It is finally collected to a clean tube and analyzed with FOXO-1 ELISA assay.

Scratch wound healing

HaCaT were seeded in 24 multi-well plates and cultured to confluence. A scratch was made in the confluent monolayer with a plastic disposable pipette tip (10 µl). Debris was removed from the culture by gently washing with sterile PBS. Hereafter, HaCaT were cultured in DMEM 10%, and treated with 10 µg/ml LPS, DD, WWB alone or in association for 24 hrs. Experiments were performed using a Nikon T-E microscope (4x objective). Cells were kept at 37°C and 5% CO_2 for all experiments. Photos were taken every 4 hrs using Metamorph software. Cell migration was measured with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health,

Bethesda, Maryland, USA). At least three fields for each condition were analyzed in each independent experiment.

Statistical analysis

Statistical significance of all experiments was evaluated by GraphPad software (Synergy Software, USA). The Dunnet's multi-comparison test was chosen because five biological replicates were done for each condition in each experiment and they were not normally distributed. Five technical replicates were performed for each experimental condition; three biological replicates were performed for each experimental condition: N=3.

Results with *p*-values < 0.05 were considered statistically significant: *p<0.05; **p<0.01; ***p<0.001.

RESULTS

WWB and DD affect cell viability of human adult keratinocytes treated with LPS.

In order to set a suitable pattern of LPS concentrations to be tested, we performed a preliminary dose-response curve of HaCaT viability. Lower concentrations of LPS (up to 10 μg/ml for 24 hrs) did not affect HaCaT number, while a higher dose (25 μg/ml for 24 hrs) significantly reduced cell viability (Fig. 1A). Based on these results, we first evaluated the effect of WWB and DD (separately or as WWB/DD complex) on cells treated with 10 μg/ml LPS (ineffective dose). High concentrations of DD significantly affected cell viability, while lower doses were ineffective (Fig. 1B). WWB (B concentration) significantly affected cell viability (Fig. 1C). The association of both compounds (WWB/DD complex) significantly reduced cell viability when added both at higher doses in HaCaT treated with 10 μg/ml LPS (Fig. 1F). Both compounds failed to prevent the cytotoxicity induced by 25 μg/ml LPS both separately (Fig. 1D, E) and in complex (Fig. 1G). For the following tests we chose the lowest, no cytotoxic concentrations of the natural compounds and we test their effects on 10 μg/ml LPS.

Release of EGF, IGF-I and KRT16.

10 μg/ml LPS promoted a significant EGF release by HaCaT (24 hrs) (Fig. 2Ai). DD and WWB, both separately and in complex, significantly prevented LPS-induced EGF release (Fig. 2Aii). The same trend was observed on IGF-1 release (Fig. 2Bi and ii). Finally, the treatment with 10 μg/ml LPS slightly, but not significantly, increased KRT16 production (Fig. 2Ci), an effect slightly prevented by DD (Fig. 2Cii).

FOXO-I distribution

Incubation with 10 μg/ml LPS (24 hrs) drastically enhanced cytosolic expression of FOXO-I (Fig. 2Di), this effect was prevented by incubation with DD and WWB, both separately or in combination (Fig. 2Dii).

Modulation of wound closure and anti-inflammatory role of WWB and DD

To evaluate the role of both compounds in the wound healing rate of HaCaT, we first examined their ability to modulate cell motility employing an established *in vitro* scratch wound healing assay. Wound closure was evaluated by observing the re-populated area between the wound margins at different time intervals (0-24 hrs) after the lesion. The wound monitoring showed that untreated cells (DMEM 10%) followed the physiological healing process, reaching approximately 65% of closure at 24 hrs after injury (Fig. 3A, Bi). 10 μg/ml LPS (24 hrs) drastically reduced wound-closure percentage, reaching approximately 35%. This effect was prevented by both DD and WWB applied separately (Fig. 3A, Bii). Application of 10 μg/ml LPS reduced VEGF production in scratched keratinocytes (Fig. 3Ci). DD and WWB/DD complex prevented the LPS effect, while WWB resulted uneffective (Fig 3Cii). Skin wounding and inflammatory responses involve cytokines that exert inhibitory activity on human keratinocyte growth. We investigated the effects of DD and WWB both separately and in association on cytokine production (IL-1 α, IL-6 and IL-8) by HaCaT upon treatment with LPS.

Incubation with 10 μg/ml LPS (24 hrs) promoted the release of IL-8, IL-1α and IL-6 (Fig. 3 Di, Ei and Fi). The lowest doses of DD and WWB, separately or in combination, did not prevent IL-1α production (Fig. 3Eii); conversely, WWB and WWB/DD were significantly effective on IL-6 and IL-8 release, while DD induced a non significant protective activity (Fig. 3Dii, Fii).

CONCLUSIONS

Acne is a multifactorial disease based on an alteration in the pattern of keratinization within the pilosebaceous follicles resulting in comedo formation, an increase in sebum production which is influenced by androgens, the proliferation of *P. Acnes*, and the development of perifollicular inflammation [19-20]. New commercially available formulations and drugs for skin treatment are increasingly introduced. Evidences suggest a potential beneficial activity of plant-derived phenolic compounds obtained either by the diet or through skin application, indeed, they can alleviate symptoms and inhibit the development of various skin disorders. Recently, a novel face compact cream (FCC) containing a new patented formulation (including both white willow bark extract and 1,2 decanediol) was developed to provide acne patients with cosmetic camouflage for their lesions and to have beneficial effects on the multifactorial components of the disease [21]. Here we tested the role of both compounds on adult keratinocytes stressed with LPS. Both compounds (used alone or in combination) counteracted LPS effects, reducing growth factor and cytokine production. They reduced EGF expression altered by the treatment with LPS. This is interesting because several studies report the alteration of EGF signaling during *acne* associated with the use of anticancer agents (eg: EGFR inhibitors). Acneiform eruptions are a common adverse reaction to EGFR inhibition and can be treated with traditional acne therapy [22]. Similarly, WWB/DD complex significantly prevented IGF-I production induced by LPS. During puberty, IGF-I signaling may have a causal role in pathogenesis of *acne* by influencing adrenal and gonadal androgen metabolism that was reported to be an inducer of sebum production through sterol response element-binding proteins [23-24]. Recently, the involvement of IGF-1, but not androgens, has been suggested in *acne* pathogenesis [25]. A relevant IGF-1-dependent mechanism that increases androgen receptor (AR) signaling involves the metabolic transcription factor FOXO-I. FOXO-I is proposed to be the key to understand the link between genetic and environmental factors in *acne*. Nuclear FOXO-I functions as an androgen receptor co-suppressor it regulates the activity of most important target genes involved in the pathogenesis of *acne* [26]. All growth factors or acneigenic stimuli mimicking

growth factor signaling might lead to the reduction of the nuclear content of FOXO-I. In the present study we observed that WWB and DD, used separately or in combination, significantly decrease FOXO-I cytosolic/nuclear ratio. Both WWB and DD slightly but not significantly prevented KRT16 expression after LPS treatment. KRT16, as well as KRT6 and KRT17, is considered a stress keratin chronically expressed in human pre-malignant hyperproliferative epithelium [27]. Finally, we tested the role of natural compounds in the modulation of keratinocyte migration and in the production of pro-inflammatory cytokines. Li M. et al., (2015) reported the ability of LPS to reduce keratinocyte migration in a diabetes-like microenvironment [28]. Here we observed that both compounds markedly promoted HaCaT migration and to enhance artificial wound closure compromised by LPS treatment, an effect related to their ability to sustain VEGF production. Cell-free extracts of *P. Acnes* significantly stimulate secretion of interleukins (IL-8 and IL-6) in SZ95 sebocytes [29], and promotes IL-8 secretion by interacting with Toll-like receptor 2 (TLR-2) [30]. IL-6 is a pleiotropic cytokine that plays a pivotal role in host defense, immune response regulation, hematopoiesis and inflammation [31]. SNPs in IL-6 gene have been identified and associated with several diseases [32-33]. However, there are relatively few studies on IL-6 gene polymorphism in *Acne* patients. It has been recently reported that the IL-6 and IL-1α gene promoter polymorphisms are associated with the pathogenesis of *acne vulgaris* [34]. IL-1α has been widely studied in inflammation and is considered to affect the pathogenesis of *acne* [35]; it also contributes to paracrine signaling between keratinocytes and fibroblasts during wound healing. WWB and DD differently affect cytokine production. Both compounds failed to counteract IL-1α release increased by LPS treatment. Conversely, WWB alone or WWB/DD complex significantly reduced IL-6 and IL-8 release, while only a slight effect could be observed upon stimulation with DD alone.

In conclusion, taken together, our results show the ability of both natural compounds to affect different functions of LPS-stressed keratinocytes.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

E.B. designed the research study, performed the research, analyzed the data and wrote the paper.
F.G. contributed essential reagents or tools and designed the research study.
L.M. designed the research study, analyzed the data, wrote the paper and contributed essential reagents or tools.
This study was funded by Rottapharm Madaus.

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FIGURE LEGENDS

Figure 1. WWB and DD exert protective effects on HaCaT treated with LPS.
A) 25 μg/ml LPS significantly reduced cell viability compared to untreated cells.

B) The highest dose of DD (A) significantly reduced cell viability of HaCaT treated with 10 μg/ml LPS. The other concentrations slightly but not significantly reduced cell viability.

C) The intermediate dose of WWB (B) significantly reduced cell viability of HaCaT treated with 10 μg/ml LPS. The other concentrations were ineffective.

D) The highest dose of DD (A) significantly reduced cell viability of HaCaT treated with 25 μg/ml LPS. The other concentrations were ineffective.

E) All the concentrations of WWB (A-C) were ineffective on cell viability of HaCaT treated with 25 μg/ml LPS.

F) The combination of the highest doses of WWB/DD (A) significantly reduced cell viability of HaCaT treated with 10 μg/ml LPS.

G) All the concentrations of WWB/DD were ineffective on cell viability of HaCaT treated with 25 μg/ml LPS. *DD (A:5.2mg/ml, B:26μg/ml, C:13μg/ml); WWB (A:520 μg/ml, B:260 μg/ml, C:130 μg/ml), WWB/DD (A/A: 5.2mg/ml+520 μg/ml; B/B: 26μg/ml + 260 μg/ml ; C/C: 13μg/ml + 130 μg/ml)*.

Figure 2. The lowest concentrations of WWB and DD differently affect EGF, IGF-I and KRT16 production and FOXO-I translocation.

A) HaCaT were stimulated with 10μg/ml LPS alone (i) or in association with the lowest concentrations (C) of WWB and DD (ii). Supernatant was collected after 24 hrs and EGF secretion

was quantified with ELISA test. 10μg/ml LPS increased EGF production (i). WWB and DD (alone or in association) significantly reduced EGF production increased by LPS treatment (24 hrs) (ii).

B) HaCaT were stimulated with 10μg/ml LPS used alone (i) or in association with the lowest concentrations (C) of WWB and DD (ii). Supernatant was collected after 24 hrs and IGF-I secretion was quantified with ELISA test. 10μg/ml LPS increased IGF-I production after 24 hrs of treatment (i). WWB/DD complex significantly reduced IGF-I production affected by LPS (24 hrs) (ii).

C) HaCaT were stimulated with 10μg/ml LPS used alone (i) or in association with the lowest concentrations (C) of WWB and DD (ii). Cytosolic extract was collected after 24 hrs and KRT16 secretion was quantified with ELISA test. 10μg/ml LPS slightly but not significantly increased KRT16 production (i). WWB or DD used alone or in combination did not significantly modify KRT16 production (24 hrs) (ii).

D) HaCaT were stimulated with 10μg/ml LPS used alone (i) or in association with the lowest concentrations (C) of WWB and DD (ii). After 24 hrs cytosolic and nuclear FOXO-I extracts were obtained and quantified with ELISA test. 10μg/ml LPS increased cytosolic/nuclear ratio of FOXO-I (i). Both WWB and DD used alone or in association (lowest dose) significantly reduced FOXO-I ratio (24 hrs) (ii). *DD (C: 13μg/ml); WWB (C:130 μg/ml), WWB/DD (C/C: 13μg/ml + 130 μg/ml).*

Figure 3. The lowest concentrations of WWB and DD differentially regulate wound closure and pro-inflammatory cytokine production.

A) Representative micrograph of keratinocytes directly (0 hrs) or 24 hrs after scraping (original magnification 4x).

B) HaCaT cells were scratched and stimulated with 10μg/ml LPS used alone or in association with WWB and DD. Percentage of wound-closure is shown as mean ± SEM of 3 independent experiments.

C) HaCaT were scratched and stimulated with 10μg/ml LPS used alone or in presence of WWB and DD (alone or in complex, both at the lowest concentration). Supernatant was collected at 24 hrs after scraping and VEGF production was quantified with ELISA test. 10μg/ml LPS significantly reduced VEGF production (i). DD alone or in complex with WWB complex significantly prevented the VEGF reduction (ii).

D) HaCaT were stimulated with 10μg/ml LPS used alone or in presence of WWB and DD (alone or in complex, both at the lowest concentration). Supernatant was collected after 24 hrs and IL-8 secretion was quantified with ELISA test. 10μg/ml LPS significantly increased IL-8 production (i). WWB alone or WWB/DD complex significantly prevented this effect (ii).

E) HaCaT were stimulated with 10μg/ml LPS used alone (i) or in presence (ii) of WWB and DD (used alone or in complex, both at the lowest concentration). Supernatant was collected after 24 hrs and IL-1α secretion was quantified with ELISA test. 10μg/ml LPS significantly increased Il-1α release (i). DD and WWB alone or in association did not modify this effect (ii).

F) HaCaT were stimulated with 10μg/ml LPS used alone (i) or in association (ii) to WWB and DD (alone or in complex, both at the lowers concentration). Supernatant was collected after 24 hrs and IL-6 secretion was quantified with ELISA test. 10μg/ml LPS significantly increased IL-6 release (i). WWB alone or in association to DD significantly prevented this effect (ii). DD (A:5.2mg/ml, B:26μg/ml, C:13μg/ml); WWB (A:520 μg/ml, B:260 μg/ml, C:130 μg/ml), DD/WWB (C/C: 13μg/ml + 130 μg/ml).