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Angelica sinensis isolate SBD.4: composition, gene expression profiling, mechanism of action and effect on wounds, in rats and humans

This report characterizes an aqueous isolate (SBD.4) of one of the most broadly used Chinese medicinal herbs, *Angelica sinensis*, from the perspective of its application in skin and wound care. SBD.4 has been chemically defined and was found to increase the strength of healed wounds in retired breeder (older) rats. Furthermore, the mechanism of action of this *Angelica sinensis* isolate was tested in the zebrafish angiogenesis model, and in human skin substitutes by DNA microarray, revealing a bioactivity profile consistent with skin repair and regeneration. When combined with several types of wound dressings, SBD.4 increased type I collagen production in human dermal fibroblasts, and when formulated in nanosilver hydrocolloid dressing, it was found effective in chronic ulcer management in humans, demonstrating that botanical high-tech wound dressings can be successfully developed to improve the treatment of chronic lesions in humans.

Key words: *Angelica sinensis*, diabetes, ulcers, skin, arginine, sucrose, standardized botanical isolate

One key difference between Eastern and Western approaches to pharmacology is the reliance on multicomponent botanical preparations in the East versus the preference for defined, synthetic drugs in the West. As much as the Western approach may be justified in most cases, it seems that the multi-stage process of chronic wound healing could benefit more from a variety of active principles, such as those found in medicinal plant isolates. It is realistic to expect that such arrays of active principles could have complementary and/or synergistic activity resulting in stimulation of wound healing and strengthening of healed tissues, not achievable by a single molecular entity. Despite this, few botanical wound healing products made it to the bedside, due, in part, to the lack of thorough scientific validation and characterization required for product commercialization.

Angelica sinensis is one of the best-known medicinal plants in Asia, used, among others, for regularization of the menstrual cycle and treatment of angina pectoris [1-3]. We hypothesized that these indications may be at least partially substantiated by a pro-angiogenic mechanism of action of a subset of Angelica's ingredients, and used an endothelial cell bioactivity-guided approach to isolate this subset. We showed that this isolate (named SBD.4) stimulates wound healing in genetically diabetic mice and in human skin grafted on SCID mice [4]. The results reported here complete the investigation of the SBD.4 isolate in terms of its composition, organism-wide mechanism of action and effect on tissue healing beyond wound closure in rats, as well as in chronic wound management in humans, under the form of a SBD.4-nanosilver hydrocolloid wound dressing.

Materials and Methods

Composition of SBD.4

SBD.4 was prepared from *Angelica sinensis* root by aqueous extraction and fractionation as described before [5].

1. Ion analysis of SBD.4 was performed by ICP-MS (HP-7500a) on 5% HNO_3-digested solution of elemental species, (100mg/100ml). Sample was introduced at 1ml/min at atmospheric pressure and pneumatically nebulized with Ar carrier gas (1.4L/min). Matrix decomposition was performed in Ar plasma (15L/min) with direct sampling analysis. Analysis was performed in full quantification mode (six data points) with a scan rate of 0.3 sec per point. Data was averaged over ten repetitions. Quantification was performed against a standard calibration curve for twenty-three elemental ions over five calibration levels made with NIST-traceable Absolute Standards.

2. Carbohydrate analysis of SBD.4 was performed using NMR, MALDI, GC-MS, HPAEC and HPLC-RI. Briefly, 1-D Proton, COSY, TOCSY, HSQC, and HMBC NMR spectra were acquired on a Varian Inova-500 MHz spectrometer at 298 °K (25 °C) using SBD.4 dissolved in D_2O.

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Chemical shifts were measured relative to HDO ($\delta = 4.77$ ppm). For MALDI, a 10 μg sample was suspended in 0.5 M dihydroxybenzoic acid spotted on and recrystallized with dihydroxybenzoic acid, then analyzed in an Applied Biosystems 4700 mass spectrometer run in the positive ion mode. Glycosyl linkage analysis was performed on depolymerized, reduced, and acetylated sample (resulting in partially methylated alditol acetates -PMAAs) using chromatography-mass spectrometry (GC-MS) as described in [6]. GC-MS was also used for glycosyl composition analysis, using the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis.

The quantification of sucrose and glucose was done by High pH-Anion-Exchange Chromatography (HPAEC) using a Dionex DX500 system equipped with a GP40 gradient pump, an ED40 electrochemical detector, a Thermo-Separation AS3500 autosampler and CarboPac PA20 (3×150 mm) analytical column with an amino trap.

3. Citric acid quantification in SBD.4 was performed on an HP 5890 GC interfaced to a 5970 MSD, using All Tech EC-1 fused silica capillary column (30 m × 0.25 mm ID).

4. Amino acid quantification of the HCl-hydrolyzed SBD.4 was performed in duplicate on Hitachi L-8800 amino acid analyzer.

5. The method for total phenolics deterimanation was Folin-Ciocalteu [7], and for gallotanin determination – as described in [8]. Ligustilides and ferulic acid were measured by HPLC following the methodology reported in [9].

Percentages of weight of different components of SBD.4 are w:w.

DNA microarrays

EpiDermFT skin substitute tissues (Mattek, Ashland, MA) were incubated in the absence or presence of SBD.4 (1 mg/mL) for 48h, afterward tissues were frozen in liquid nitrogen and subjected to RNA extraction with Qiagen kit. The quality of extracted RNA was validated twice by electrophoresis (after extraction and before microarray analysis).

Samples were hybridized and analyzed in technical duplicates using human OneArray platform from Phalanx Biotech (Palo Alto, CA). Array data were normalized using the Quantile normalization method (http://bmbolstad.com/stuff/qnorm.pdf). Data was further processed with Array Studio V2.5 (Omicsoft) to eliminate differences with high p values ($p > 0.05$) and low fold change (< 1.5). Correction for multiple hypothesis testing was performed using the Benjamini-Hochberg method. Furthermore, all genes described as "uncategorized" and "putative" were excluded from the final analysis. Finally, Genesifter (Geospiza, Seattle, WA) software was used to identify functional categories affected by the test material.

Zebrafish angiogenesis model

Transgenic zebrafish line Tg (*fli1:EGFP*) that expresses GFP in endothelial cells was kindly provided by ZFIN (Eugene, OR) and maintained as described in the Zebrafish Handbook [10]. Embryos were generated by natural pairwise mating (3-12 months old) and were raised at 28.5°C in embryo water. Twenty four hours post fecundation (hpf) zebrafish embryos were collected, distributed into a 12-well microplate with 10 fish in each well and pretreated with 100 ng/mL VRI (VEGFR tyrosine kinase inhibitor II, Calbiochem, La Jolla, CA, cat. #676481) for 5 h. VRI was then replaced with different concentrations (10-500 μg/mL) of SBD.4 (dissolved at 100 mg/mL in DMSO and further diluted in Milli-Q water) for 24 h. Embryos treated with 0.1% DMSO was vehicle control, equivalent to no treatment. All experiments were repeated 3 times, with 10 embryos per group.

The effect of SBD.4 on angiogenesis was quantified by counting the defective and intact intersegmental vessels (ISVs) in each embryo, as described before [11]. Briefly, VRI was used to inhibit ISV elongation from dorsal aorta (DA), caudal artery (CA) and posterior cardinal vein (PCV), then this inhibition was challenged by different concentrations of SBD.4. Morphological changes proportional to the angiostimulatory effect were assessed 24 h later using Olympus Spinning Disk Confocal Microscope setup (IX81 Motorized Inverted Microscope w/ZDC, IX2 universal control box, X-cite series 120, DP71 CCD camera).

Healing-impaired rat incisional wound model

Male rats (Sprague-Dawley retired breeders 12 months old) were purchased from Charles River Laboratories. On Day 0, rats were anesthesized, randomly divided in groups of 4 and injected intramuscularly with 5mg methylprednisolone suspension in saline to induce glucocorticoid impairment, which results in delayed wound healing [12]. Four incisional wounds (2cm each) were then performed on each rat, 2 at each side of the dorsal median divide.

SBD.4 isolate was formulated at 20 mg/mL in 1% carboxymethylcellulose (CMC) and becaplermin (BCP, the positive control) was used directly from the tube. Test materials were applied at 100 μL (2 mg) / wound, while the control animals were treated with 1% CMC. Immediately after injury and test material application the wounds were sutured by one suture in the center of the incision. The animals were sacrificed on Day 13, wounds were then excised and fixed in formalin. The median strips 0.5 cm × 1.6 cm were excised, washed for 24h in PBS and mounted in pneumatic grips in an Instron Universal Testing Apparatus with a 0.4 inch gauge length. Pulling force was applied at 1.0 inch/min. crosshead speed, and the force required to break wounds was measured. The remaining portions of the wounded tissues were mounted in paraffin and sectioned from the median in 5 μm increments perpendicularly to the surface of the wound, then deparaffinized and stained with Masson's trichrome stain. The reported data are averages from 2 independent experiments with different sets of animals and different batches of SBD.4 isolate used each time.

Animal experiments were conducted in a licensed facility (MMRI, Sunnyvale, CA) and complied with the US Public Health Service Policy on the Human Care and Use of Laboratory Animals and all of the applicable provisions of the Animal Welfare Act, and the US Government Principles for the Utilization and Care of Vertebrate Animals in Testing, Research and Training (USDA). The animal experimentation protocol for this project was reviewed and approved by the institutional Animal Care and Use Committee (IACUC).

Effect of SBD.4 on type I & III collagen in conditioned media of neonatal human dermal fibroblasts (HDF) incubated with different wound dressings

Aqueous wound dressing extracts were prepared by incubating samples of commercial dressings – Prisma, lot #1322771 (Johnson & Johnson – J&J, New Brunswick, NJ); Promogran, lot #2995690 (J&J); Silvasorb, lot #05101902 (Medline Industries, Mundelein, Il); and Sureskin II, lot B0801022 (Euromed, Orangebourg, NY) at 5 mg/mL for 3 h under constant agitation in the presence or absence of 5 mg/mL SBD.4. The insolubilities were then eliminated by centrifugation and the supernatants were added to HDF (ATCC, lot #PCS-201-010, passage 6) at 1/20 dilution. After 4 days of incubation, type I & III collagen in the cell-conditioned medium was measured by sandwich enzyme-linked immunoabsorbant assay (ELISA), as reported before [13, 14].

Antimicrobial activity determination and clinical case studies of SBD.4-nanosilver hydrocolloid wound dressing

SBD.4 was formulated at 1% in a silver-containing commercial hydrocolloid dressing (Sureskin Silver) by Euromed, Inc. and its antimicrobial activity was evaluated by the Time to Kill/Log Reduction assay [35]. Dressings were pre-soaked in sterile water for 24h, then inoculated with suspensions of microorganisms listed in *table 5* in 5% bovine serum for specified contact time at 20 °C afterward plate count test was carried out to enumerate the surviving organisms. Dey Engley broth was used as neutralizer.
In clinical case studies, sterile SBD.4 dressings were applied on lower extremity chronic ulcers in 4 patients under a protocol approved by an Institutional Review Board (IRC, San Anselmo, CA, protocol #09125-01) in the medical clinics of Dr. K. Hara (Santa Paula, CA) and Dr. F. Vagnini (Lake Success, NY), until the ulcers healed (3 weeks to 3 months). All patients were suffering from type II diabetes and had non-healing lower extremity ulcers since at least 3 months.

Statistical analysis

Data are presented as the mean determinants (± SEM). Paired Student t-test was used for statistical analysis and differences with p values < 0.05 were considered statistically significant, except for the rat wound model, where the Tukey's multiple comparison test was applied.

Results

Composition of the SBD.4 isolate

Table 1 provides the elemental composition of SBD.4 obtained by ICP-MS. Of interest are the high concentrations of phosphorus (5.3 g/kg), calcium (3.8 g/kg) and magnesium (2.8 g/kg) as well as low concentrations of heavy metals, such as lead (0.06 ppm) and mercury (below 0.01 ppm).

Table 1. Elemental composition of SBD.4 isolate from *Angelica sinensis* by ICP-MS showing phosphorus as the most prominent element, followed by calcium and magnesium. Results reported in mg/kg as average of 10 measurements ± standard deviation.

Analyte	Report result	Standard deviation (+/-)
Na	1390	51.5
P	5289	184.7
Ca	3769	181.3
Ti	2.67	0.19
Fe	48.70	1.3
Mo	0.27	0.02
Sn	0.13	0.01
Be	<0.01	
Mg	2811	122.3
Al	9.51	0.33
V	0.17	0.01
Cr	0.12	0.03
Mn	17.17	0.57
Co	<0.01	
Ni	0.39	0.03
Cu	1.89	0.10
Zn	39.93	2.09
As	0.14	0.03
Se	0.23	0.02
Ag	0.07	0.03
Cd	0.02	0.01
Sb	0.02	0.01
Ba	3.69	0.14
Hg	<0.01	
Tl	0.02	0.00
Pb	0.06	0.01

The carbohydrate analysis of SBD.4 by NMR detected sucrose and arginine at an approximate ratio 4:1 *(table 2),* and MALDI detected ions consistent with NMR, plus citric acid. Linkage analysis established that the vast majority of glycosyl r residues in SBD.4 are terminal in nature, which is consistent with the presence of high levels of sucrose, and citric acid was quantified by GC-MS at 5 g/kg. The monosaccharide composition of SBD.4 was further analyzed by HPAEC, which revealed sucrose and glucose at ratio 5:1, as well as traces of galactose *(figure 1).* The total carbohydrate amount in SBD.4 was established at 744 g/kg. The amino acid analysis of SBD.4 confirmed the prevalence of arginine observed by NMR and MALDI, and established its percentile at 65 of total amino acids. The other amino acids detected were glutamine/glutamate (10%), proline (8%), alanine (5%), glycine (5%), asparagine/aspartate (3.3%), histidine (3.3%), all other amino acids combined making less than 0.5%. The total amino acid component was established at 160 g/kg of the isolate.
Finally, total phenolics were determined at 7.5 g/kg (as gallic acid), of which 82% was found to be gallotannins and 6% free gallic acid. Ligustilides and ferulic acid combined were found to be less than 0.5 g/kg.

Table 2. Proton and carbon chemical shifts of SBD.4 detected by NMR as compared with literature values, establishing the presence of sucrose and arginine in this isolate at a 4:1 ratio.

Compound	Residue		Chemical Shift (ppm)					
SBD.4			1	2	3	4	5	6
Sucrose	α-Glc*p*	^{1}H	5.41	3.55	3.75	3.46	3.84	3.81
		^{13}C	*93.1*	*72.5*	*73.9*	*70.6*	*73.2*	*61.3*
	β-Fru*f*	^{1}H	3.67		4.21	4.05	3.89	3.81
		^{13}C	*63.2*	*104.6*	*77.9*	*75.2*	*82.5*	*63.2*
Arginine		^{1}H		3.76	1.90/1.90	1.71/1.66	3.23/3.23	
		^{13}C	*175.8*	*55.3*	*28.7*	*25.4*	*42.0*	*158.0*
Literature								
Sucrose[a,b]	α-Glc*p*	^{1}H	5.42	3.56	3.77	3.47	3.90	n.d.
		^{13}C	*92.9*	*72.0*	*73.6*	*70.2*	*73.3*	*61.1*
	β-Fru*f*	^{1}H	3.69		4.22	4.05	3.86	n.d.
		^{13}C	*63.3*	*104.4*	*77.4*	*75.0*	*82.2*	*63.4*
Arginine[c,d]		^{1}H		3.79	1.93/1.93	1.72/1.72	3.27/3.27	
		^{13}C	*175.2*	*55.6*	*28.8*	*25.2*	*41.7*	*157.8*

[a] *Proton: Tarelli and Wheeler. Carbohydr. Res. 1995 ; 269 : 359*
[b] *Carbon: Pfeffer et al. J. Am. Chem. Soc. 1979 ; 101 : 1265*
[c] *Proton: Ziegler et al. J. Magn. Res. 1996 ; 112 : 141*
[d] *Carbon: Surprenant et al. J. Magn. Res. 1980 ; 40 : 231*

DNA microarrays

DNA microarray analysis was performed on human full thickness skin substitutes treated by 1mg/ml SBD.4 for 48 h, to gain insight into the molecular mechanisms underlying the previously observed wound healing effect of SBD.4. *Table 3* contains a list of genes relevant to wound healing, which were found to be modulated by SBD.4, with their brief description. *Table 4* organizes these and other genes into functional groups (ontologies). Predictably, strong stimulation of collagens and laminins, along with a weaker increase of expression of hyaluronate synthase, hb-EGF and claudins, as well as the decrease of ADAM9 protease, translates into enhanced cell adhesion, reinforced extracellular matrix, growth factor and nitric oxide (NO) signal transduction, generally consistent with a skin- and wound-healing activity of SBD.4 [15].

In contrast, no clear angiomodulatory information could be deduced from the DNA microarray analysis and therefore, in order to confirm or disprove our previous findings concerning the angiostimulatory potential of SBD.4 *in vitro*, we tested it in the zebrafish angiogenesis model.

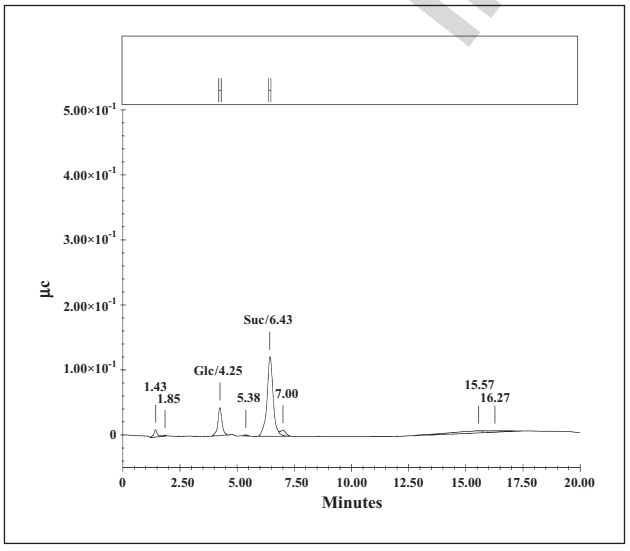

Figure 1. HPAEC chromatogram of SBD.4 on CarboPac PA 20 column, using water/100 mM NaOH gradient elution and ED4 electrochemical detector, showing the predominance of sucrose (Suc) and glucose (Glc).

Zebrafish angiogenesis model

Pretreatment of 24 hpf transgenic zebrafish line Tg (fli1:EGFP) that expresses GFP in endothelial cells with 300 nM VEGFR tyrosine kinase inhibitor VRI for 5 h reduced intersegmental blood vessels (ISV) and impaired DLAV (dorsal longitudinal anastomotic vessels) formation *(Fig. 2B).* As illustrated in *figure 2* and quantified in *figure 3*, SBD.4 induced angiogenesis in a dose-dependent manner in this model, achieving a statistically-significant improvement already at the lowest dose tested (10 µg/ml).

Healing-impaired rat incisional wound model

Based on the DNA microarray results showing an increase of the fibril-associated type XVI collagen expression, our previously published increase of type I collagen output and our current finding on type III collagen stimulation, we hypothesized that wounds treated with SBD.4 may convert into a stronger healed tissue as compared with controls. To test this hypothesis, 100 µl of 2 mg/wound SBD.4, BCP or CMC control was applied on 2 cm incisional wounds performed on glucocorticoid-impaired retired breeder Sprague-Dawley rats and the wounds were

Table 3. Wound-relevant genes modulated by 48 h incubation with 1 mg/mL SBD.4 in EpiDermFT tissue substitutes (DNA microarray analysis).

Gene Name	Protein Product	Modulation (up- down)	Protein Role
COL17A1	Collagen XVII	4.2 up	Major structural component of hemidesmosome, anchoring basal epithelial cells to the underlying basement membrane.
COL16A1	Collagen XVI	3.2 up	Fibril-associated collagen with interrupted triple helix important for tensile strength of the skin.
HAS3	Hyaluronan synthase 3	1.7 up	Synthesizes the most prominent non-proteinaceous component of the extracellular matrix in the skin.
LAMC2, LAMA3	Laminin γ-2 Laminin 5	3.9 up 2.6 up	Key component of the basement membrane (basal lamina), laminin 5 is involved in cell adhesion, signal transduction and differentiation of keratinocytes and laminin γ-2 interacts with laminin 5 to form anchoring filaments that connect epithelial cells to the underlying basement membrane. Laminin γ-2 is epithelium-specific.
SOD2	Superoxide dismutase 2	1.7 up	Mitochondrial Mn-dependant SOD-2 provides an important anti-oxidant activity, especially in tissues exposed to increased oxygen and free radical presence, such as in the wounds.
ADAM9	ADAM 9	1.7 down	Disintegrin metalloprotease interacting with type I collagen and implicated in delayed wound healing.
HBEGF	Heparin-binding EGF	1.6 up	Epidermal growth factor involved in wound healing and other regenerative processes in the skin.
CLDN1 CLDN4	Claudin 1 Claudin 4	2.0 up 2.0 up	Prominent components of the tight junctions, where they establish the paracellular barrier that controls the flow of molecules in the intercellular space between the cells of an epithelium.

Table 4. Gene ontologies (GO) relevant to skin and wound healing, modulated by 48 h incubation with 1 mg/ml SBD.4 in EpiDermFT skin substitutes (DNA microarray analysis data processed by Genesifter software).

Cell adhesion/ extracellular matrix/skin/wound healing						
Ontology	**List**	**Up**	**Down**	**Array**	**z-up**	**z-down**
Cell adhesion mediated by integrin	1	1	0	2	2.57	-0.4
Heterophilic cell adhesion	3	3	0	5	5	-0.63
Cell-substrate junction assembly	3	3	0	4	5.72	-0.57
Positive regulation of tissue remodeling	2	2	0	8	2.21	-0.8
Cell-cell adhesion	15	13	2	92	3.19	-1.94
Extracellular matrix organization and biogenesis	2	2	0	6	2.76	-0.7
Cell junction assembly/organization	3	3	0	11	2.91	-0.94
Calcium-dependent cell-matrix adhesion	1	1	0	1	3.90	-0.28
Collagen fibril organization	2	2	0	4	3.64	-0.57
Heparan sulfate proteoglycan biosynthetic process, polysaccharide chain biosynthetic process	1	1	0	2	2.57	-0.4
Platelet-derived growth factor receptor signaling pathway	2	2	0	5	3.14	-0.63
Establishment of cell polarity	2	2	0	6	2.76	-0.7
Nitric oxide mediated signal transduction	2	2	0	4	3.64	-0.57

"List" indicates how many genes in a given ontology group are significantly modulated by SBD.4; "Up" and "Down" determines the direction of the modulation; "Array" informs how many genes in total make the given GO group; "z-score" indicates whether modulation in a specific GO group appears more or less frequently than expected by chance. Positive numbers greater than 2 indicate that it occurs more frequently than expected and thus it is enhanced, while values less than -2 indicate the contrary.

allowed to heal for 13 days. The histological sections of the closed wounds revealed differences in SBD.4 treated- and untreated animals. Specifically, the control wounds contained a large area of amorphous granulation tissue with few basket weave- type collagen fibers (*figure 4A*), while in SBD.4-treated specimens, this amorphous tissue was reduced and replaced by robust collagen bundles (*figure 4B*). In order to determine whether these morpho-

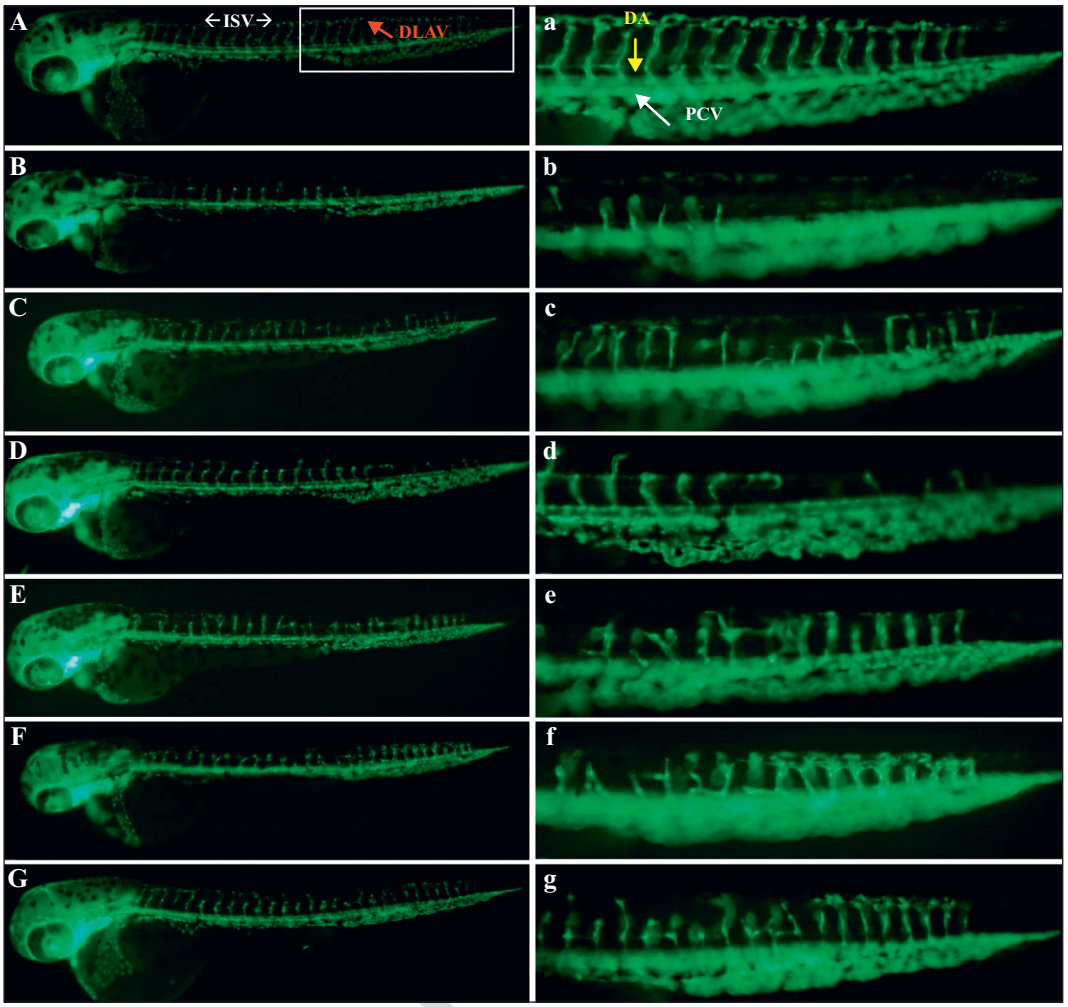

Figure 2. Pro-angiogenic effect of SBD.4 in zebrafish embryos.
Low **(A-G)** and high **(a-g)** power images of developing vasculature in zebrafish, box in **(A)** indicates the magnified views of **(A-G)**. **A)** Control: live fluorescence microscopy highlights EGFPexpressing intersegmental blood vessels (ISV) (white double arrow) and dorsal longitudinal anastomotic vessels (DLAV) (red plain arrow); **B)** VRI-induced inhibition of ISV and DLAV; **C-F)** VRI-pretreated zebrafish embryos, treated with 10 μg/mL SBD.4 **(C)**, 30 μg/mL SBD.4 **(D)**, 100 μg/mL SBD.4 **(E)**, 300 μg/mL SBD.4 **(F)**, 500 μg/mL SBD.4 **(G)**, showing dose-dependent angiostimulatory effect of SBD.4. PCV: posterior cardinal vein; DA: dorsal aorta.

logical differences translate into stronger tissue, median portions of wounds were excised and their tensile parameters were quantified by tensiometry. *Figure 4C* shows that both the average peeling load (in Newtons) and the average peeling strength (in newtons [N]/cm^2) were doubled in SBD.4-treated animals, confirming the histological observations, while becaplermin had no effect.

Effect of SBD.4 in wound dressing extracts on type I & III collagen output in HDF populations.

The results above were encouraging enough to consider formulating SBD.4 in wound dressings for human use. To determine whether such formulation could benefit healing wounds, we incubated HDFs with extracts of several wound dressings prepared in the presence or absence of SBD.4. *Figure 5* shows that the inclusion of SBD.4 improved type I collagen output by HDF populations incubated with extracts prepared from dressings with (Prisma, Silvasorb) or without silver (Promogran, Sureskin II). Similarly, the Angelica isolate had a dose-dependent, statistically-significant stimulatory effect on type III collagen levels in adult HDFs, with no effect at 50 μg/mL and a peak stimulation at 1 000 μg/mL of 43% (not shown).

Antimicrobial activity determination and clinical case studies with SBD.4-nanosilver hydrocolloid wound dressings.

Faced with the choice of wound dressing matrices to formulate with, we opted for a nanosilver hydrocolloid, which would allow the combination of the wound-healing activity of SBD.4 with the anti-microbial effect of silver and the absorbing, protecting and debridement-promoting properties of the hydrocolloid matrix. Accordingly, the resulting

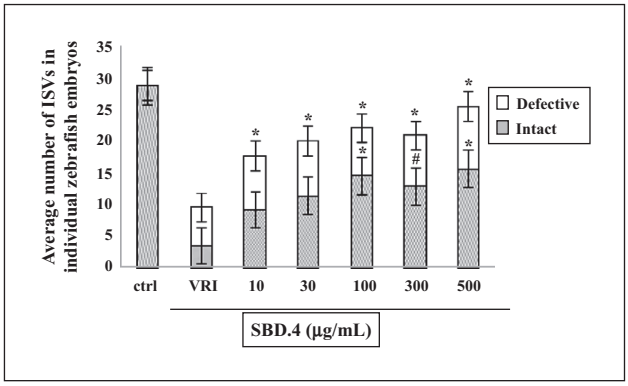

Figure 3. Quantification of ISV formation in zebrafish embryos from *figure 2*. Number of defective and intact ISVs in each embryo was counted in a minimum of 10 embryos per group over a series of 3 independent experiments, after 24 h treatment with SBD.4. *p < 0.01 *vs* VRI treatment group. #p < 0.05 *vs* VRI treatment group.

dressing, formulated by Euromed using their Sureskin nanosilver hydrocolloid platform, was found to have a broad antimicrobial profile *in vitro (table 5*). It was over 99.9 % effective in killing P.aeruginosa and C.albicans starting at 6 h and 24 h respectively, and it was 93% effective against MRSA at 72 hrs of contact time.

The sterile SBD.4 dressing was then tested under the IRB-approved protocol on 4 chronic lower extremity ulcers. All patients' wounds healed while applying the experimental SBD.4 dressing. *Figure 6* shows a typical ulcer at the beginning (Day 0) and the end (Day 30) of the treatment.

Discussion

Traditional herbal medicines have been used to treat chronic diseases since the dawn of medical practice. However, the reverse of their tremendous overall contribution to public health has been their complexity and lack of standardization (for example see [16]). Here, we thoroughly describe the composition of an aqueous isolate with wound healing activity from *Angelica sinensis*.

Skin- and wound-healing precedence can be established for every major component of the SBD.4 isolate. Sucrose, by far the most abundant part of the extract, is an easily assimilated source of energy for tissue/cellular regeneration, possesses antimicrobial activity and its use in wound dressings is widely documented [17-20]. Arginine, the second most abundant component, is a precursor of nitric oxide, the deficiency of which has been established as a major causative factor in diabetic ulcers [21]. Accordingly, arginine-containing wound dressings have activity in diabetic wound healing [22]. Arginine may also contribute to skin moisturization (as a precursor of urea), mitochondrial metabolism (as a precursor of creatine) and enhanced extracellular matrix formation, by interconvertion to proline – an amino acid critical for maintaining the conformational stability of collagens. Proline itself and glutamine (precursor of antioxidant glutathione) are also prominently represented in SBD.4, as is glycine, which constitutes 30% of collagen's amino acid sequence. Of interest is also

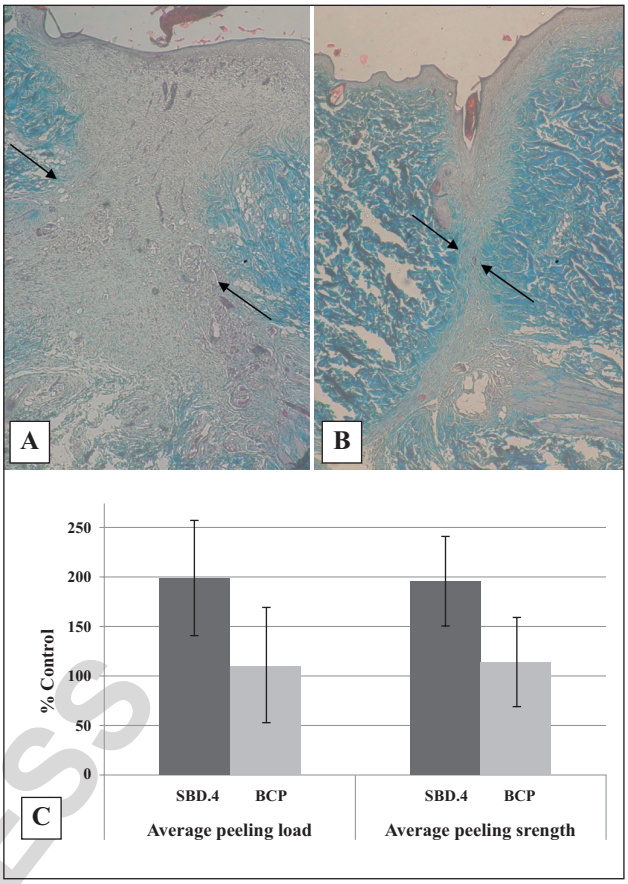

Figure 4. Effect of SBD.4 on the incisional wound healing in older, glucocorticoid-impaired rats.**A, B)** Trichrome stains of closed wounds. Control-treated **(A)** shows less organized collagen (blue stain) and more amorphous tissue than the SBD.4-treated **(B)** wounds (see arrows). Original mag.: × 40. **C)** Quantification of average peeling load and average peeling strength in wounds treated by SBD.4 (2 mg/wound) and becaplermin (BCP) expressed as % of vehicle (carboxymethylcellulose)-treated control. Wounds closed in the presence of SBD.4 appear to be stronger as compared with BCP and control.

citric acid – an alpha-hydroxy acid (AHA) indispensable for energy generation through Krebs cycle in the mitochondrium. Besides having buffer and preservative functions, citric acid induces the epidermis to remodel and accelerate desquamation [23], as well as increases viable epidermal thickness and glycosaminoglycan content of sun-damaged skin – effects similar to retinoic acid [24]. Among the inorganic components, phosphorus and calcium have been found to be the most abundant. Inorganic phosphorus is key structural (DNA, RNA, proteins, phospholipids), signal transduction (phosphorylation/dephosphorylation) and energy transfer (ADP/ATP) component of life. Increased P concentration is particularly important for diabetic wounds, where ATP level is depressed [25]. The normal concentration of Ca in the epidermis increases from 0.5mM at the basal layer to 1.4mM in the stratum granulosum, proportionally to the increased chance of damage. This is because calcium is essential for wound repair, where it is predominantly involved as Factor IV in the hemostatic phase and in epidermal cell migration and fibroblast proliferation at later

Table 5. Growth inhibition of *S. aureus*, *P. aeruginosa* and *C. albicans* at different time points by nanosilver SBD.4-hydrocolloid wound dressing as compared with control (no dressing), by Time Kill Test/Log Reduction Assay, over a period of 72 h. Organism suspensions of 10^6 cfu/mL in 5% bovine serum were inoculated onto the presoaked product for specified contact time and plate count tests were carried out to enumerate the surviving organisms [35].

Microorganism	Organism Initial count	Control (no dressing)					Nanosilver SBD.4-hydrocolloid wound dressing				
		0 h	6 h	24 h	48 h	72 h	0 h	6 h	24 h	48 h	72 h
S. aureus (MRSA) ATCC # 43300	2.8×10^5	2.9×10^3	6.8×10^5	3.4×10^7	3.9×10^7	9.0×10^7	2.9×10^5	2.2×10^5	1.7×10^5	2.1×10^4	2.02×10^4
% Reduction		0%	0%	0%	0%	0%	0%	21%	39%	92.4%	93%
Log Reduction		0	0	0	0	0	0.01	0.11	0.22	1.13	1.14
P. aeruginosa ATCC # 9027	9.9×10^6	9.7×10^6	5.5×10^6	3.00×10^7	3.20×10^7	3.40×10^8	9.9×10^6	2.10×10^3	2.6×10^2	0	0
% Reduction		0%	0%	0%	0%	0%	0%	99.98%	99.99%	>99.99%	>99.99%
Log Reduction		0	0	0	0	0	0	3.674	4.581	6.996	6.996
C. albicans ATCC #10231	1.1×10^5	1.10×10^5	2.3×10^5	1.80×10^6	1.1×10^6	3.3×10^6	1.2×10^5	2.5×10^4	1.0×10^2	0	0
% Reduction		0%	0%	0%	0%	0%	0%	77.3%	99.91%	>99.99%	>99.99%
Log Reduction		0	0	0	0	0	-0.04	0.642	3.04	5.04	5.04

stages [26]. Accordingly, calcium alginate wound dressings were engineered to slowly release Ca to the wound bed. Taken together, this composition analysis demonstrates that a small molecular weight, water soluble fraction of *Angelica sinensis* has wound-healing relevant bioactivites and it raises the possibility that it may be responsible for some of the biological effects previously attributed to Angelica polysaccharides [27]. The fact that this isolate contains individual amino acids and monosacharides rather than proteins and oligo- or polysaccharides demonstrates the capacity of simple elements in a multicomponent preparation to combine their disparate effects into one powerful therapeutic activity.

DNA microarray experiments showed that the SBD.4 isolate enhances collagen XVI & XVII expression, which translates into enhanced collagen fibril organization, and together with the ADAM9 decrease may explain the stimulation of collagen deposition by HDF observed in this and previous studies [4, 15].

Similarly, the increase of HAS3 expression is consistent with our previous finding of SBD.4 enhancing hyaluronic acid production by the same cells [28]. Furthermore, the stimulation of nitric oxide-mediated signal transduction pathways, critical for chronic wound healing [21] is in agreement with the high content of arginine (precursor of NO) in SBD.4 isolate. Taken together, the DNA microarray data indicate that SBD.4 promotes wound healing through multiplex effects on extracellular matrix components, mediated by growth factors and nitric oxide, which results in improved dermal/epidermal structure.

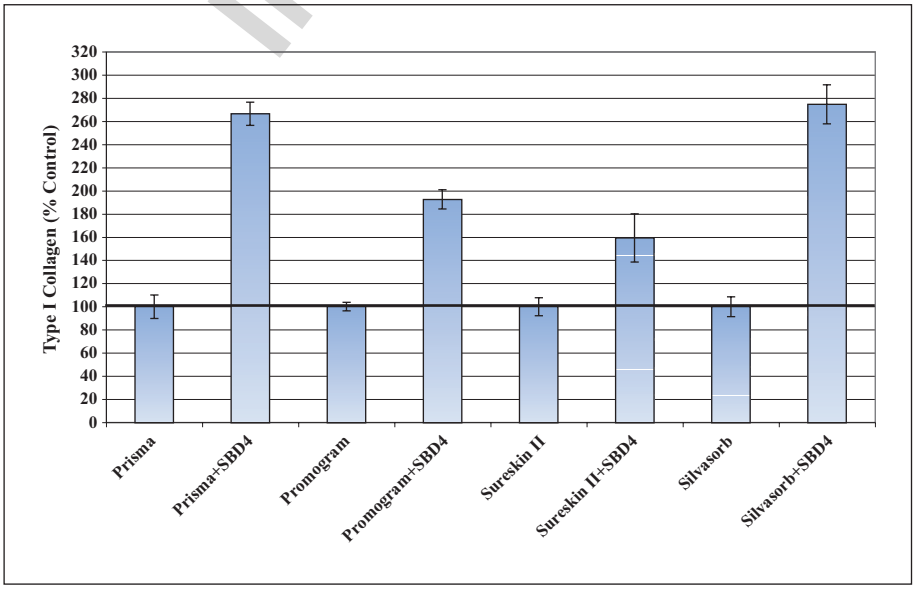

Figure 5. Type I collagen deposition by human dermal fibroblasts in the presence of 4 different wound dressing extracts prepared without or with SBD.4. Addition of SBD.4 at 5 mg/mL resulted in the increase of collagen deposition in all 4 cases as measured by sandwich ELISA after 4 days of incubation.

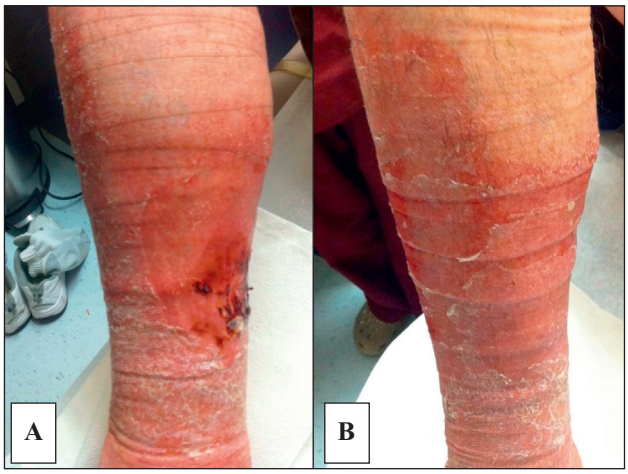

Figure 6. Typical effect of SBD.4-nanosilver hydrocolloid (Euromed, Inc.) hybrid wound dressing (4 in × 4 in) on chronic ulcers. Non-healing diabetic leg ulcer on treatment Day 0 (A) and treatment Day 30 (B).

One type of information missing from the microarray data was the effect of SBD.4 on angiogenesis, perhaps due to the lack of blood vessel components in the EpiDermFT tissue substitutes. Therefore, in order to confirm or disprove our *in vitro* data suggesting the pro-angiogenic effect of SBD.4, we tested it in a whole organism, vasculature-compromised zebrafish model. Zebrafish (Danio rerio) has emerged as a powerful model in drug screening. Many cardiovascular, angiogenic, and anticancer treatments, including botanical extracts, elicit similar responses in zebrafish embryos as in mammalian systems [29, 30]. The finding that SBD.4 isolate elicits a strong, dose-dependent proangiogenic response in vasculature-compromised zebrafish confirms our *in vitro* data [4, 28] and the results obtained with another *Angelica sinensis* extracts [31, 32]. It also underlines the importance of precise extract standardization, as other extracts from the same plant were reported to have an opposite effect [33, 34].

SBD.4 isolate was found to enhance excisional wound healing in diabetic mice and in human skin implants on SCID mice [4]. The DNA microarray data obtained here was consistent with this activity. Furthermore, the upregulation of type III and XVI collagens, which associate with type I collagen and provide strength to extracellular matrix fibrils in the dermis and muscles, prompted us to determine the effect of SBD.4 on the strength of healed wounds. This is an important issue, as chronic wounds are not only hard to heal but also easy to break and reopen under a physical load. SBD.4 was found to double the resistance of the healed wounds to tearing (although, interestingly, only in older rats - the results in young rats not shown), providing further rationale for the use of SBD.4 in wound dressings.

Inclusion of SBD.4 in several wound dressing extracts in order to study the resulting effect on type I collagen was the next step towards the final product formulation. Addition of SBD.4 resulted in the increase of type I collagen output by HDF. This increase may be due either to stimulation of fibroblast proliferation, stimulation of type I collagen synthesis, inhibition of collagen-digesting proteases, stabilization of type I collagen fibril structure or a combination thereof. The DNA microarray and type III collagen ELISA data support the inhibition of a protease (ADAM 9) and stabilization of microfibrils (through upregulation of type III and XVI collagen) hypotheses, although more research is needed to determine the exact mechanism of this stimulation.

The last step of the development of SBD.4 into a wound-active product was its formulation into a nanosilver hydrocolloid dressing (Sureskin Silver) for human use. The choice of the dressing matrix was dictated by the objective to create an easily manageable, inexpensive product with a broad application range (moderately discharging ulcers) with an added antimicrobial support. Our preliminary data show that the resulting product is well tolerated over prolonged periods of use (months) and in all four cases its use was concomitant with complete healing of refractory ulcers.

Given the overall results, it is concluded that SBD.4 isolate of *Angelica sinensis* formulated in wound dressings may provide a significant improvement in wound treatment, bridging the best of the Eastern and Western treatment modalities for the benefit of patients with chronic ulcers. ■

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