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Inhibition of *Pseudomonas aeruginosa* biofilm formation on wound dressings

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Abstract

Chronic non-healing skin wounds often contain bacterial biofilms that prevent normal wound healing and closure and present challenges to the use of conventional wound dressings. We investigated inhibition of *Pseudomonas aeruginosa* biofilm formation, a common pathogen of chronic skin wounds, on a commercially available biological wound dressing. Building upon prior reports, we examined whether the amino acid tryptophan would inhibit *P. aeruginosa* biofilm formation on the 3-dimensional surface of the biological dressing. Bacterial biomass and biofilm polysaccharides were quantified using crystal violet staining or an enzyme linked lectin, respectively. Bacterial cells and biofilm matrix adherent to the wound dressing were visualized through scanning electron microscopy. D-/L-tryptophan inhibited *P. aeruginosa* biofilm formation on the wound dressing in a dose dependent manner and was not directly cytotoxic to immortalized human keratinocytes although there was some reduction in cellular metabolism or enzymatic activity. More importantly, D-/L-tryptophan did not impair wound healing in a splinted skin wound murine model. Furthermore, wound closure was improved when D-/L-tryptophan treated wound dressing with *P. aeruginosa* biofilms were compared with untreated dressings. These

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findings indicate that tryptophan may prove useful for integration into wound dressings to inhibit biofilm formation and promote wound healing.

Keywords

Biofilm; Wound Dressing; Tryptophan

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Introduction

Bacterial biofilms account for nearly 80% of chronic infections, according to the CDC (1). A 2008 study revealed that 60% of chronic skin wounds (i.e. pressure, venous leg, and diabetic foot ulcers) contained bacterial biofilms (2). Biofilms significantly delay normal healing by serving as a reservoir of bacterial cells that resist both antibiotic treatment and clearance by the immune system (3–7). This bacterial burden can trigger a chronic inflammatory state and thus disrupt the normal healing process. The 'gold standard' for chronic skin wound treatment is to debride the wound to remove necrotic tissue and microorganisms in order to create a clean wound bed (8). However, bacterial biofilm remaining in the wound may resist the debridement process and serve as a resistant reservoir of bacterial cells leading to additional delays in healing. Novel treatments that disperse or prevent biofilm formation are required to improve the care of chronic non-healing skin wounds (2).

Wound dressings can provide additional surface area for bacterial cell attachment and biofilm formation within chronic wounds (9–11). Various reports have documented the presence of bacterial biofilms on wound dressings and a variety of medical implants, including surgical sutures and wound dressings using scanning electron microscopy (SEM) (12–14). Contamination of wound dressings often necessitates their removal and leads to additional wound debridement, increasing patient discomfort and further delaying the healing process (15–18). There is a pressing clinical need for innovative approaches to prevent adherence of bacterial cells and formation of biofilms on the surfaces of implants, sutures, and wound dressings. Strategies currently employed include chemical modification of the dressing to limit bacterial attachment and controlled release of antibiotics to eliminate planktonic bacterial cells (19–21). An alternative and complimentary strategy is to target the biofilm directly. Incorporating compounds that inhibit and disperse biofilms in the wound dressing itself may help prevent biofilm growth on the dressing and wound bed and therefore help improve healing.

Biofilm inhibitors described in the literature include antimicrobial peptides, metal chelators, quorum sensing inhibitors, and amino acids (22–27). Inhibition of bacterial biofilm growth with D-amino acids was first reported for the gram-positive species *Bacillus subtilis* and *Staphylococcus aureus* (28, 29). Inhibition was initially attributed to disruption of the peptidoglycan cell wall, and destabilization of the protein – matrix interaction, but later was credited to disruption of protein synthesis in *B. subtilis* (30). The amino acid tryptophan has been reported to inhibit biofilm formation by the gram-negative pathogens *Escherichia coli* (31) and *Pseudomonas aeruginosa* (32). Our laboratory demonstrated that D- and L-isoforms of tryptophan both inhibited *P. aeruginosa* biofilm formation and dispersed existing *P.*

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aeruginosa biofilms within 24 hours of treatment. Although the mechanism responsible for biofilm inhibition and dispersal by tryptophan remains uncertain, it may involve increased bacterial motility or altered quorum sensing (33–36). An added advantage of using tryptophan as a biofilm inhibitor in chronic wounds is the recently described beneficial effect it has on wound healing and closure (37–39).

One limitation of many *in vitro* biofilm studies is reliance on a relatively simple 2-dimensional abiotic surface, such as polystyrene microtiter plates, that does not reflect the complexity of biofilms in the wound environment. To investigate inhibition of biofilm formation on complex surfaces, such as within a chronic skin wound, we established a model for *P. aeruginosa* biofilm formation on a commercially available biological wound dressing (Biobrane). Biobrane was chosen for its complex 3-dimensional geometry and synthetic/biological heterogeneity (40). Using this model system we show that tryptophan dose dependently inhibits biofilm formation on a biological wound dressing. In addition, we demonstrate the absence of *in vitro* cytotoxicity of tryptophan using two different immortalized human keratinocyte cell lines and observed no deleterious effects when tryptophan was applied topically to experimental full thickness mouse skin wounds. We also demonstrated the potential *in vivo* benefit of using tryptophan to inhibit *P. aeruginosa* biofilm formation on the wound dressings using the same full thickness murine skin wound model. These studies provide evidence for the continued exploration and development of tryptophan as an anti-biofilm agent for treatment of chronic skin wounds.

Materials and Methods

Bacterial Strains and Materials

Pseudomonas aeruginosa American Type Culture Collection (ATCC) strain 27853 was used in all experiments. Bacto™ Tryptic Soy Broth (TSB) (Becton, Dickinson, and Company, Sparks, MD) and M63 minimal media (2.0g $(NH_4)SO_4$, 13.6g KH_2PO_4, 0.5mg $FeSO_4$•$7H_2O$, 10ml 20% glycerol, and 1ml 1M $MgSO_4$ in 1.0L of diH_2O, pH~7.0) were used for overnight bacterial growth and biofilm experiments, respectively. Saturated solutions of 50 mM D- and L-isoforms of tryptophan (Sigma-Aldrich, St. Louis; Acros Organics, New Jersey) were prepared in 1% Phosphate Buffered Saline (PBS) and filter sterilized using a 0.22μm syringe filter. The wound dressing, Biobrane, was purchased from UDL Laboratories Inc. (Rockford, IL). An 8 mm biopsy punch was used to cut the dressings into discs, which were aseptically placed into separate wells of 48 well microtiter plates for biofilm inhibition and dispersal experiments.

Quantification of Biofilm Formation and Dispersal

P. aeruginosa was incubated overnight (~24h) at 37°C under rotation until a concentration of approximately 10^9 CFU/ml was obtained. The overnight culture of *P. aeruginosa* was inoculated into the M63 minimal media at a 1:2500 dilution with or without and equimolar ratio of D- and L-tryptophan (0.5 – 10mM) prior to addition to the wound dressings. For dispersal experiments, 48 hour old biofilms were formed on the dressings in the M63 minimal media without tryptophan at 30°C under static conditions. After 48 hours of growth, planktonic bacterial cells were removed by rinsing the dressings 3 times with sterile

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1X PBS. Fresh M63 minimal media containing Tryptophan (0 – 10mM) was then added to the dressings for 24 hours at 30°C prior to biofilm quantification by crystal violet or enumeration of colony forming units (CFU).

To quantify biofilm mass, the dressings were washed 4 times with deionized H_2O, air dried, stained with 0.35% filtered crystal violet (EMD Chemicals, Gibbstown, NJ) for 15 minutes, and washed 5 times with tap water as previously reported (32, 41). Bound crystal violet was solubilized with 30% Acetic Acid and solution absorbance measured at 595nm using a microplate reader (Beckman Coulter 880DX). The amount of bound crystal violet was proportional to the bacterial biomass on the surface of the wound dressings and expressed as the mean + the standard error of the mean (SEM) absorbance at 595nm. In some experiments wound dressings were stained with a phosphatase-linked lectin instead of crystal violet. To do so, the wound dressings were washed 3 times with sterile normal saline (0.9% w/v) prior to incubation with alkaline phosphatase conjugated Hippeastrum hybrid lectin (Amaryllis) (E-Y Laboratories, San Mateo, CA) for 18 hours at 4°C. This lectin was chosen because it has been shown to bind extracellular polysaccharide produced in *P. aeruginosa* biofilms (42). Excess lectin was removed from the wound dressings by washing 3 times with sterile normal saline. The amount of lectin bound to the biofilm was quantified via colorimetric decay of P-nitrophenylphosphate (colorless) to P-nitrophenol (yellow color) by the conjugated phosphatase at a solution absorbance of 405nm. To quantify CFU, the dressings were rinsed 4 times with 1X PBS and placed in sterile Eppendorf tubes containing 1.0ml of sterile 1X PBS and Zirconium Oxide beads. The samples were homogenized with a Bullet Blender® (Next Advance, Averill Park NY), diluted and plated on trypticase soy agar with 5% sheep's blood, and incubated at 37°C for 18 – 20 hours. Colonies were counted and the values expressed as the mean + SEM $\log_{10}$ (CFU/dressing). Each experimental group was performed in triplicate and repeated for three independent experiments.

Scanning Electron Microscopy

Wound dressings were bathed with *P. aeruginosa* suspended in M63 minimal media (~5×10^5 CFU/ml) with or without 10.0mM D-/L-tryptophan for up to 72 hours at 30°C. At 24, 48, and 72 hours the dressings were washed with 1X PBS and fixed with 4% (v/v) paraformaldehyde (Electron Microscopy Sciences) for 20 minutes at room temperature. Following fixation the samples were washed with PBS and stored in 2.5% (v/v) gluteraldhyde /1% (v/v) tannic acid at 4°C until stained with 0.5% (v/v) Osmium Tetroxide for 30 minutes. The dressings underwent a series ethanol dehydration and critical point drying with CO_2. Samples were mounted onto scanning electron microscope stubs and sputter coated with platin/carbon. Images were acquired at a magnification of 5000× and a working distance of ~9mm using a LEO 1530 scanning electron microscope.

In vitro cytotoxicity

To measure acute cytotoxicity, D-tryptophan was dissolved in Dulbecco's phosphate buffered saline (DPBS, with Ca2+ and Mg2+, pH 7.4, Thermo Scientific, Logan, UT) at a concentration of 10 mM and serially diluted in DPBS to final concentrations of 1.0 nM – 10.0 mM. Prior to plating cells, 96-well tissue culture plates were coated with 40 µl of fibronectin-collagen mix (FNC, AthenaES, Baltimore, MD) for 15 minutes. The

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immortalized human keratinocyte cell line, HaCaTs were seeded in the FNC coated 96-well microtiter plates at ~5×10^5 cells/well in 200 μl of DMEM/Endothelial Cell Basal Medium EBM-2 (Lonza, Walkersville, MD) and allowed to adhere overnight. Control conditions included cells treated with 200 μl of normal cell culture medium (positive control), DPBS (vehicle control), or 3% saponin solution (negative control) in DPBS. After a 1 hour incubation period at 37°C in 5% CO2, cells were rinsed 3 times with DPBS. Each well then was incubated for 30 to 60 minutes with 200 μl of calcein-AM (Invitrogen, Eugene, Oregon) solution (1 μM in DPBS). Fluorescence (Ex: 485nm; Em: 528nm) was measured using a Synergy 4 Hybrid Microplate Reader with Gen5 software (Biotek, Winooski, VT). Tryptophan treated wells were expressed as the mean ± SEM percentage of the DPBS control wells and the assay was repeated in triplicate.

Skin hTERT immortalized normal keratinocytes (STINKs) derived from neonatal human foreskin were kindly provided by Dr. Vladimir S. Speigelman (Department of Dermatology, UW-Madison) and used for 24 and 48 hour cytotoxicity studies. The STINKs were grown in Eagle's minimum essential medium (EMEM) with nonessential amino acids and L-glutamine without Calcium (Lonza) supplemented with Epidermal Growth Factor (EGF) at 5ng/ml, Bovine Pancreas Insulin at 5μg/ml, Transferrin at 10ng/ml, Ethanolamine at 10μM, Phospho-ethanolamine at 10μM, Calcium Chloride at 20μM (Sigma), 7% Fetal Bovine Serum (Atlanta Biologicals), and 1% Penicillin-streptomycin-amphotericin B (Corning) at 37°C with 5% CO_2 (43). Cells were split using 0.25% Trypsin without EDTA (Corning). To measure cell viability and cytotoxicity the RealTime-Glo™ MT Cell Viability Assay (Promega) was multiplexed with CellTox™ Green Cytotoxicity Assay (Promega) and were used according to the manufacturer instructions. Briefly, opaque (white) 96 well plates (NUNC) were seeded with 10,000 STINKs cells/well and allowed to adhere overnight. The media was aspirated and replaced with complete culture medium containing the RealTime-Glo reagents and tryptophan (0 – 10mM). The RealTime-Glo™ MT Cell Viability Assay contains a substrate that is reduced within metabolically active cells to the substrate for the NanoLuc® luciferase and the resulting luminescence is measured. The CellTox™ Green Cytotoxicity Assay contains a cyanine dye that is excluded from viable cells but stains DNA in lysed or membrane permeable cells. Luminescence, the indicator of cellular metabolism and viability, was measured at 24 and 48 hours post tryptophan treatment and expressed as the mean ± SEM Luminescence Units. Thirty minutes before adding the CellTox™ Green Reagent, lysis buffer was added to a set of control wells to determine the maximum fluorescence of dead cells. Once the Cell-Tox Green Reagent was added to the wells, the plates were incubated for 15 minutes at room temperature. Fluorescence, the indicator of cell lysis or death, was measured at EX:485nm/EM:535nm and expressed as the mean ± SEM Relative Fluorescence Units (RFU). The multiplexed assay was repeated four times in triplicate.

Animal Model

To assess the impact of tryptophan on wound healing we utilized an experimental murine skin wound model, previously established in our laboratory (44, 45). All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison. Eight to twelve week old BALB/c mice were housed

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individually in a temperature-controlled facility with a standard light/dark cycle. Environmental enrichment, food, and water ad libitum were provided to all mice. Mice were anaesthetized with isoflurane (Piramal Healthcare, Bethlehem, PA) using an induction chamber. Buprenorphine (0.1 mg/kg) was administered for pain control. The cranial thoracodorsal region was shaved and aseptically prepared for surgery. Silicon O-rings (McMaster-Carr®) were secured with tissue glue (Tissumend II) to the skin 4 mm caudal to the base of the ears on each side of the dorsal midline and were further attached to the skin by six 5-0 interrupted nylon sutures. Two symmetrical wounds within each O-ring were made using a 6mm biopsy punch. Body weights of mice were recorded on post-operative day 1, and every 2–3 days until the end of the study. Upon completion of each study, mice were euthanized by intra-peritoneal injection of Beuthanasia®-D (Schering-Plough) solution (0.5 ml/mouse) after induction of anesthesia with isoflurane.

In vivo wound impairment study

To measure the effects of tryptophan on wound healing, twenty BALB/c mice were randomly assigned to one of 4 treatment groups using a permuted block randomization of block size 4. The treatments consisted of two 8mm diameter circular Kendall Telfa Pads (Tyco Healthcare Group, Mansfield, MA), cut with an 8mm biopsy punch, stacked on top of each other and saturated with 60μl of either A) 1% PBS, B) 50mM D-Tryptophan, C) 50mM L-Tryptophan, or D) 25mM D-/25mM L-tryptophan (46). Every wound was also covered by Tegaderm™ Film (3M Healthcare, St. Paul, MN). Both wounds on a single mouse received the same treatment. The 50 mM concentration was chosen because it is the maximum tryptophan concentration achievable in aqueous media. The surgical team was blinded to the group allocation. Treatments were applied on the day of the surgery and reapplied on days 3 and 6 post surgery. Mice recovered from anesthesia on a warming pad. Each wound was imaged using a Nikon D300 digital camera with an attached Micro-Nikkor lens (105mm f/2.8G) on days 0, 3, 6, and 9 and wound areas calculated. Wound areas from the sequential images were traced and measured by 3 independent technicians blinded to the treatment groups using NIH Image J. The average calculated wound size was normalized to its own baseline (day 0) and wound area remaining on days 3, 6, and 9 was determined.

Effect of inhibiting biofilms with tryptophan *in vivo*

To determine if inhibition of *P. aeruginosa* biofilm formation by tryptophan provided an *in vivo* benefit, the wound dressings were incubated in suspensions of *P. aeruginosa* in M63 minimal media with and without 10mM D-/L-tryptophan at 30°C for 48 hours. After 48 hours of incubation the dressings were rinsed three times with 1X PBS to remove planktonic bacterial cells and applied to 6mm diameter skin wounds in mice using the same method as previously described. Immediately prior to dressing application, 18 mice were randomly assigned to one of three treatment groups using a permuted block randomization of block size 3. The treatment groups included: 1) dressings without bacterial cells (Control), 2) dressing with intact *P. aeruginosa* biofilm, and 3) dressing with tryptophan inhibited biofilm. Ten millimeter diameter Kendall Telfa Pads circular cutouts were placed on top of the wound dressing to ensure sustained contact of the dressing with the wound. The wounds were additionally covered with Tegaderm™ Film. Both wounds on each mouse received the same treatment. The surgical team was blinded to the group allocation. On days 3, 6, and 9

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post-wounding, 2 mice from each group were euthanized and the wounds imaged to determine the percentage of original wound remaining as previously described, biopsies of the wounds and dressings were taken for quantitative bacteriology. The wound areas and bacterial counts are the expressed as the mean ± SEM of two independent experiments.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey's post-test using GraphPad Prism 5.02 software (GraphPad Software, La Jolla, CA). Significance was set at $p<0.05$.

Results

P. aeruginosa readily formed biofilms on the biological wound dressing within 48 hours of incubation *in vitro* at 30°C. Figure 1A shows representative macroscopic images of wound dressings stained with crystal violet. At 48 hours biofilm growth was readily apparent on the control samples (top left panel of 1A) as compared to an uninoculated control dressing (bottom left panel of 1A). An equimolar ratio of D- and L-tryptophan at a total concentration of 10.0mM significantly inhibited biofilm formation on the wound dressing (top right panel of 1A). Tryptophan alone did not significantly affect background staining of the dressing incubated in bacteriologic broth without bacteria (bottom right panel of 1A). Two independent methods were used to quantify the amount of biofilm formed on the dressing. Figure 1B shows significantly more crystal violet stain solubilized from the control wound dressings than those incubated with 10mM D-L-tryptophan ($p<0.05$). A phosphatase-linked lectin specific for the polysaccharides present in *P. aeruginosa* biofilms confirmed the presence of biofilm matrix on the dressings. Figure 1C shows significantly increased amounts of bound lectin on the control dressings compared to those incubated with tryptophan ($p<0.05$). Both assays produced similar results with biomass (crystal violet) correlating with polysaccharide (lectin) and a relatively low background signal of the dressing itself in both assays.

We used scanning electron microscopy to visualize both bacterial cells and biofilm matrix adherent to the collagen coated nylon fibers and silicon backing of the dressing. Representative images of the dressings incubated in *P. aeruginosa* with and without tryptophan are shown in Figures 2 and 3. Figure 2 shows *P. aeruginosa* attached to the collagen-coated nylon fibers on control (Figures 2A, 2C, and 2E) and tryptophan-treated (Figures 2B, 2D, and 2F) dressings. Figure 3 shows *P. aeruginosa* attached to the silicon backing of the dressing in control (Figures 3A, 3C, and 3E) and tryptophan-treated (Figures 3B, 3D, and 3F) dressings. At 24 hours of incubation, bacterial cells are adherent to both the fibers and silicon backing of the dressing (Figures 2A/B and 3A/B). After 48 hours, bacterial aggregates or microcolonies were observed on the dressing (Figures 2C/D and 3C/D). Bacterial cells incubated with the dressing in the presence of tryptophan did not exhibit the same well-defined microcolony structure as the controls (Figures 3C vs 3D). At 72 hours, only single bacterial cells were observed on the fibers of the dressing, although microcolonies were still present on the silicon backing (Figures 2E/F and 3E/F).

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We further characterized the dose response of tryptophan on *P. aeruginosa* numbers and biofilm biomass. Figure 4 shows significant inhibition by D-/L-tryptophan concentrations above 5mM of biofilm formation as assessed by crystal violet staining and CFU counts, after 48 hours of incubation in M63 minimal media ($p<0.05$). Figure 5 shows that tryptophan did not significantly disperse 48 hour old *P. aeruginosa* biofilms from the wound dressing, although 5 and 10mM tryptophan concentrations limited additional biofilm growth as determined by both crystal violet staining and CFU counts ($p<0.05$).

We examined whether the millimolar tryptophan concentrations effective for inhibiting *P. aeruginosa* biofilms would hinder wound healing or cause cytotoxic effects on mammalian cells *in vitro*. Figure 6A shows that no significant differences in wound area remaining were observed among four treatment groups (PBS, 50mM D-tryptophan, 50mM L-tryptophan, and 50mM D-/L-tryptophan) over 9 days in a splinted mouse skin wound model. Additionally, no significant side-effects (e.g. weight loss, pain, or irritation) were observed in any mice with any of the tryptophan treatments. To fully characterize any potential toxicity of tryptophan, we used two immortalized human keratinocyte cell lines to examine acute and long term toxicity *in vitro*. Figure 6B shows that acute exposure of the immortalized human keratinocyte HaCaT cell line to D-tryptophan (1nm – 10mM) for 1 hour resulted in no change in cellular metabolism or viability. We used immortalized keratinocytes from human foreskin (STINKs) to characterize longer term toxicity of tryptophan. Figure 6C shows that for longer incubations (24 and 48 hours) D-/L-tryptophan was not cytotoxic to the keratinocytes as seen by the lack of fluorescence from DNA staining, but Figure 6D indicates that tryptophan did alter the metabolic activity of the STINKs cells.

To determine if tryptophan inhibition of *P. aeruginosa* biofilm formation may provide an *in vivo* benefit, we applied contaminated wound dressings to experimental mouse skin wounds. Representative images of the wounds on days 0 and 9 are shown in Figure 7A. Figure 7B shows that control dressings with *P. aeruginosa* biofilms exhibited no wound closure at any time over the 9 days of the study, whereas the sterile dressings showed approximately 50% healing and the tryptophan inhibited biofilm dressings showed approximately 35% healing on day 9. Figure 7C shows that there was slightly fewer bacterial cells on the dressing treated with 10mM D-/L-tryptophan compared to the untreated group ($p<0.05$) on the day of application, although after 6 days, bacterial counts on the dressings in both the tryptophan-treated and control groups reached comparable levels. Figure 7D shows the bacterial numbers isolated from the wound tissue itself. Significantly fewer bacterial cells were recovered from the wound tissue with the tryptophan-treated biofilms on day 9 compared to the control biofilms.

Discussion

Wound dressings and surgical sutures often become contaminated with bacterial cells that can form biofilms on the surface of the wound dressing or implant (9–13). These biofilms can serve as a nidus of infection leading to exacerbation of the wound and delayed healing. While their development and persistence is multifactorial, it is clear that chronic non-healing skin wounds are often infected with pathogenic biofilms that impact the healing process (9,

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47). The overall focus of our research team is to develop novel biomaterial-based dressings, which can be applied to chronic non-healing skin wounds, to prevent formation of or eliminate bacterial biofilms on the skin wound bed and aid healing. In the current study we documented that the essential amino acid tryptophan inhibits *P. aeruginosa* biofilms on a clinically relevant biological wound dressing. We further characterized any potential cytotoxicity of tryptophan both *in vitro* and *in vivo* at biofilm inhibitory concentrations, and showed this biofilm inhibition potentially may benefit wound healing.

We previously reported that an equimolar ratio of D- and L-tryptophan inhibited biofilm formation by *P. aeruginosa* on polystyrene microtiter plates (32). Although plastic microtiter plates are a standard model for studying bacterial biofilm formation, they do not replicate the complex surfaces biofilms grow on in skin wounds or the dressings used to treat such wounds. We developed a model system for studying biofilm inhibition on a complex 3-dimensional substrate by using the biological wound dressing Biobrane. This wound dressing, which is commonly used to treat skin wounds such as burns, is composed of porcine collagen-coated nylon fibers embedded in a sheet of silicon. The dressing is designed to promote re-epithelialization of the wound surface via a scaffolding effect of the collagen-coated fibers (40). Contamination of the dressing with bacterial cells often leads to its failure and requires replacement (15–18). We chose to use *P. aeruginosa* because it is commonly isolated from skin wound infections and the complex 3-dimensional geometry and biological heterogeneity of the biological dressing is a surface that *P. aeruginosa* may encounter within wounds. We believe this approach provides a realistic surface for modeling biofilm formation and inhibition on materials used to treat chronic skin wounds.

Macroscopically, *P. aeruginosa* (ATCC 27853) formed robust amounts of biofilm on the surface of the wound dressing as quantified with crystal violet and enzyme linked lectin staining. Scanning electron microscopy revealed that the bacterial cells attached to both the nylon fibers and the silicon backing of the dressing. It also showed the typical 'life-cycle' of *P. aeruginosa* biofilms in terms of attachment, microcolony formation, and potentially dispersal. Overall, we observed more bacterial cells attached to the silicon backing than the nylon fibers, although more work is required to determine if *P. aeruginosa* preferentially attaches to silicon versus the collagen-coated fibers. Incubation of wound dressings with 5mM or greater concentrations of D-/L-tryptophan significantly arrested biofilm formation. Microscopy and CFU data suggested that tryptophan may reduce attachment of *P. aeruginosa* cells to the dressing surfaces (both the nylon fibers and silicon backing), but further quantitative studies are necessary to confirm this observation. Scanning electron microscopy also revealed that tryptophan may inhibit or delay microcolony formation on the silicon backing of the dressing. Our previous work showed that tryptophan increased the swimming motility of *P. aeruginosa*, indicating that increased flagellar activity may result in decreased biofilm formation (32). Based on the present and previous experimental data, perhaps tryptophan limits bacterial attachment or disrupts microcolony formation through altered bacterial motility. Another potential mechanism for the observed effects of tryptophan may be altered quorum sensing, which could alter bacterial attachment and biofilm formation. Tryptophan feeds into the Pseudomonas quinolone signal (PQS) quorum sensing pathway and may result in altered production of biofilm matrix material through

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modulation of gene expression (33, 48). Additional studies are in progress to address the mechanism of action through which tryptophan inhibits biofilm formation by *P. aeruginosa*.

The millimolar concentration required for tryptophan to inhibit *P. aeruginosa* biofilms raised concerns regarding potentially cytotoxic effects against mammalian cells if tryptophan were to be incorporated into topical skin wound treatments. To address this concern, we performed both *in vitro* cell viability and *in vivo* wound healing studies. Acute exposure of HaCaT cells, an immortalized human keratinocyte cell line, to 10.0mM D-tryptophan for 1 hour did not reduce cell viability compared to controls, indicating that tryptophan was not directly cytotoxic to mammalian cells. We used an additional immortalized keratinocyte cell line from neonatal human foreskin (STINKs) for longer term *in vitro* cell viability tests. Tryptophan did not cause cell lysis or death but did alter or reduce the cellular metabolism of the STINKs cells, potentially through effects on the aryl hydrocarbon receptor (39).

For *in vivo* experiments, we utilized a blinded, randomized, placebo-controlled trial to compare the effects of tryptophan on wound healing in a splinted mouse skin wound model. We chose this experimental design to eliminate potential bias of animal selection and treatment bias by the surgical team in the study. We asked two separate questions regarding the effect of tryptophan *in vivo*. The first was if tryptophan would impair wound healing or cause any systemic side effects, thus limiting its applicability as an antibiofilm agent for chronic non-healing skin wounds. We chose a much higher concentration of tryptophan, 50mM, to best evaluate safety of the topical application of tryptophan and determine if there were negative outcomes to its application. This concentration would also provide a margin of safety as it is greater than the concentration required to inhibit *P. aeruginosa* biofilm formation. No significant differences in wound healing during a 9 day period were observed between control wounds and those treated with tryptophan. In addition, no other systemic side-effects (e.g. weight loss, discomfort, etc.) were observed in any of the study animals, thereby establishing the potential safety of using tryptophan at millimolar concentrations for biofilm treatments in chronic skin wounds. The second question we asked was whether inhibiting *P. aeruginosa* biofilms with tryptophan provides any benefit *in vivo*. We bathed wound dressings for 48 hours in *P. aeruginosa* with or without tryptophan and applied the contaminated dressings to murine skin wounds and assessed wound closure and bacterial counts on the dressing and wound tissue over 9 days. Most interestingly, the wounds that received the control biofilms remained 100% open over the 9 days of the experiment. The wounds, which received dressings on which biofilm formation was inhibited by exposure to tryptophan, remained approximately 65% open over the 9 days. Comparatively, the wounds that received sterile dressings were approximately 50% open after 9 days. We are currently pursuing application of biofilms on wound dressings as a potential model for developing a chronic non-healing skin wound in animal studies.

Other recent evidence supports using tryptophan as a novel wound healing agent. Tryptophan is metabolized in mammalian cells by the enzyme Indoleamine 2,3-dioxygenase (IDO) 1, which converts it into N-formylkynurenine. Mice lacking this enzyme had faster rates of healing than their wild type counterparts, coupled with increased mRNA levels of Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), and Matrix Metallopeptidase 9 (MMP9) at wound sites (37). Furthermore, it was shown in the same set of studies that the

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wild type mice treated with tryptophan had faster rates of healing than control wild type mice, as well as increased IL-6, TNF-α, and MMP9 expression in the wound tissue (37). The effect of tryptophan on wound healing was also observed in chronically stressed mice which exhibited a reduction in Collagen III formation, increased Collagen I deposition, and increased re-epithelialization of the wound (38). The beneficial aspects of tryptophan in wound healing also led to a human clinical trial of application of 50mM L-tryptophan to chronic leg ulcers (39). The tryptophan treated groups had a faster rate of wound healing, which was speculated to be modulated through activation of the aryl hydrocarbon receptor (39). Additionally, the patient group treated with tryptophan reported decreased amounts of pain compared to the control group. Furthermore, levels of systemic tryptophan are reported to be decreased in patients with chronic wounds; while the tryptophan metabolism enzymes IDO and Kynureminase (converts 3-hydroxy-L-kynurenine into 3-hydroxyanthlanilic acid) are reported to be up-regulated at sites of atopic dermatitis and psoriasis (49, 50).

A limitation of our studies is the lack of a defined system or delivery vehicle for releasing tryptophan in a controlled manner. Tryptophan was delivered as a bulk reagent in the *in vitro* experiments and Telfa® pads were saturated with tryptophan for the *in vivo* wound healing experiment. Saturated dressings were chosen for their relative ease of manipulation, but do not represent an ideal vehicle for delivery or release of tryptophan to the wound surface. In a separate study we utilized a hyaluronic acid gel for application of tryptophan to the mouse skin wounds and did not observe any detrimental side-effects or impairment of wound healing (data not shown). Ongoing studies are aimed at incorporation of tryptophan into novel polyvinyl alcohol - polyelectrolyte multilayer (PVA-PEMs) microfilms that can release tryptophan in a controlled and sustained manner. Because these microfilms can be directly applied to the skin wound bed to release both antimicrobials and antibiofilm agents into the wound microenvironment, it is hoped they may enhance the overall bio-activity of tryptophan. By releasing tryptophan from the PEMs directly into the local wound microenvironment it may be possible to overcome the millimolar concentration of tryptophan required when provided in bulk solution *in vitro*. We previously observed similar effects of increased bioactivity with silver loaded PEMs (20, 44, 45). We believe it is possible to modulate release of tryptophan from these PVA-PEMs to allow sustained release over time so that its beneficial effects may be extended beyond the time of application.

In summary, this study demonstrates that the amino acid tryptophan dose dependently inhibits *P. aeruginosa* biofilm formation on the complex three-dimensional surface of a biological wound dressing currently used for treatment of skin wounds. This system serves as a model for studying biofilm inhibition on a relevant substrate often encountered by the pathogen *P. aeruginosa* in the wound environment. We also show that tryptophan was not directly cytotoxic to immortalized human keratinocytes *in vitro*. Additionally, application of tryptophan to murine skin wounds did not inhibit wound closure nor show any toxic side-effects. Recent reports in the literature provide additional evidence that tryptophan is a promising candidate for improving wound healing. The ability of tryptophan to inhibit bacterial biofilm formation, coupled with its lack of toxicity make it a good candidate for development as an antibiofilm treatment for chronic non-healing skin wounds.

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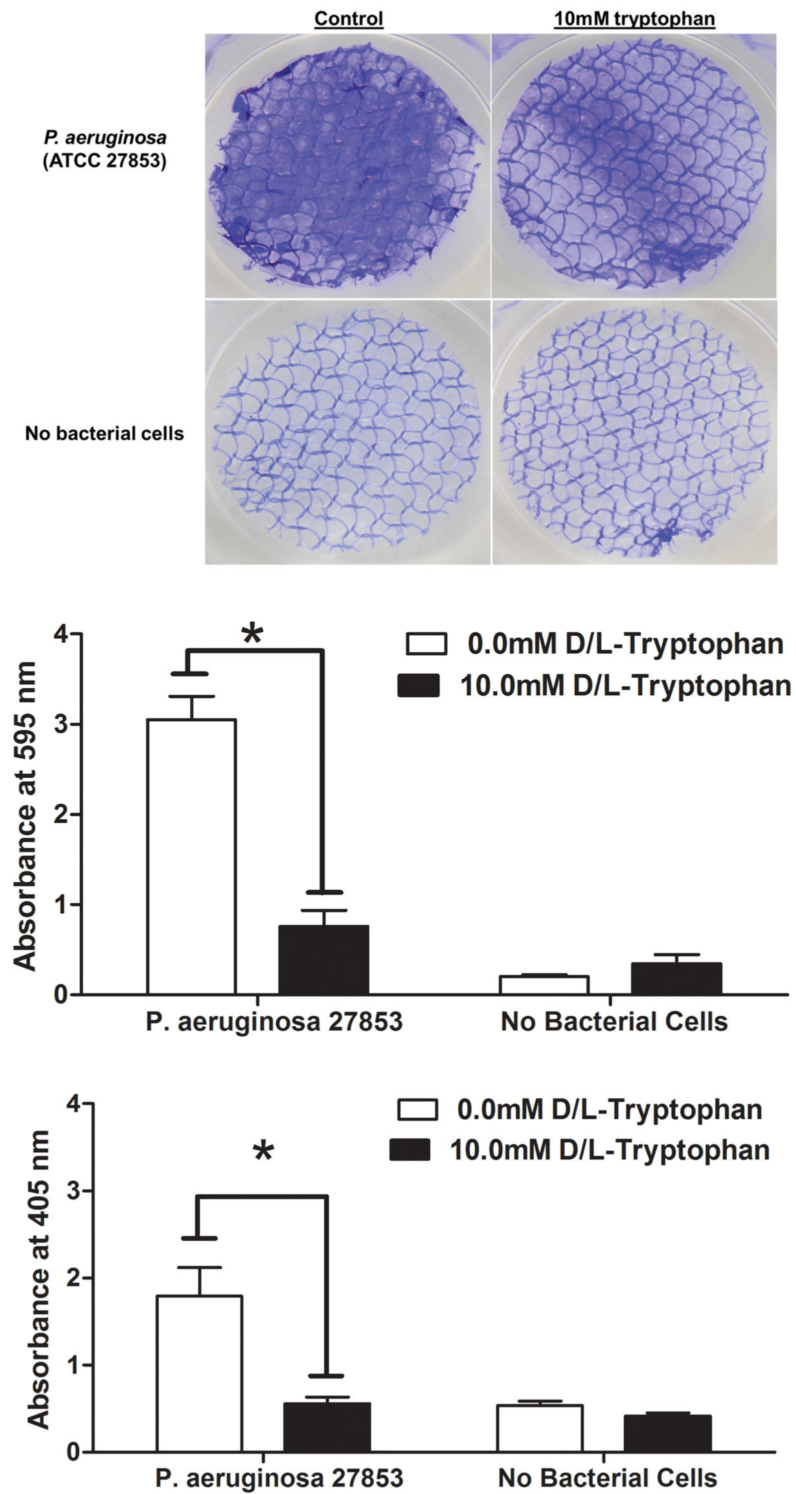

Figure 1. Tryptophan inhibits *P. aeruginosa* biofilm formation on a biological wound dressing

A) Representative samples of eight millimeter diameter sections of the wound dressing incubated for 48 hours at 30°C with *P. aeruginosa* (ATCC 27853) suspended in M63 minimal media, with or without 10mM D-/L-tryptophan. Crystal violet stained the biofilm

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on the dressing; samples incubated without bacterial cells were included as controls for background staining of the dressing. B) Solubilized crystal violet bound to the biofilm was quantified at a solution absorbance of 595nm. Tryptophan significantly inhibited biofilm growth on the wound dressing (*, $p<0.001$). C) A phosphatase linked lectin (HHA) specific for the polysaccharides of *P. aeruginosa* biofilms stained the biofilm matrix on the dressing. Biofilms were quantified by enzymatic cleavage of P-nitrophenylphosphate to P-nitrophenol at an absorbance of 405nm. Tryptophan significantly inhibited biofilm growth on the wound dressing (*, $p=0.0032$). Data is presented as the mean ± SEM of three independent experiments performed in triplicate.

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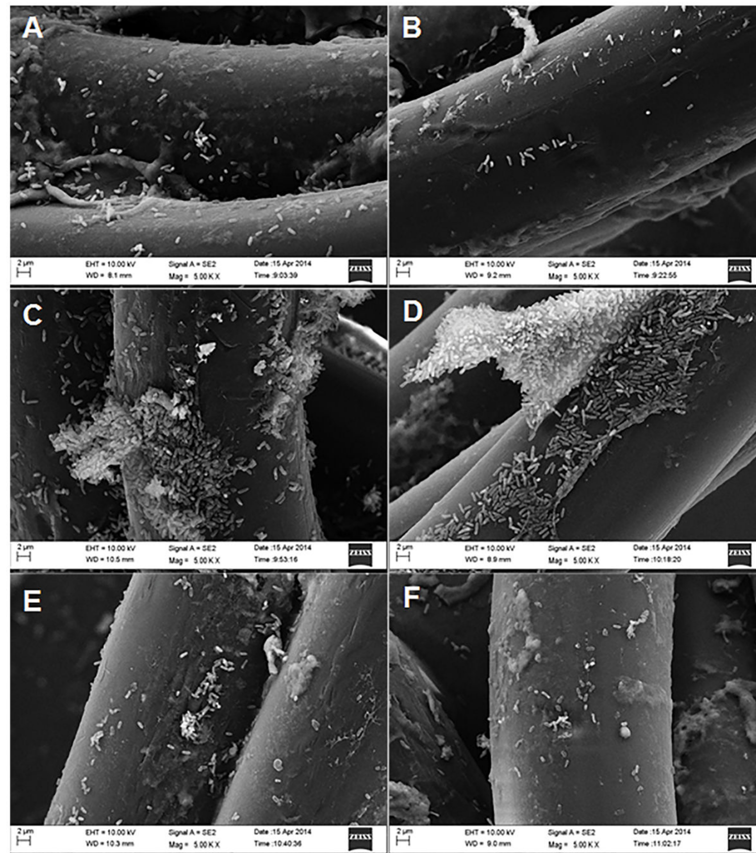

Figure 2. Scanning electron microscopy of *P. aeruginosa* biofilms on collagen coated nylon fibers of a biological wound dressing

Representative scanning electron microscopy images (5000X) of control *P. aeruginosa* biofilms (A, C, and E), and 10mM D-/L-tryptophan inhibited biofilms (B, D, and F) grown on the wound dressing for 24 h (A and B), 48 h (C and D), and 72 h (E and F). Images were taken with a LEO 1530 scanning electron microscope.

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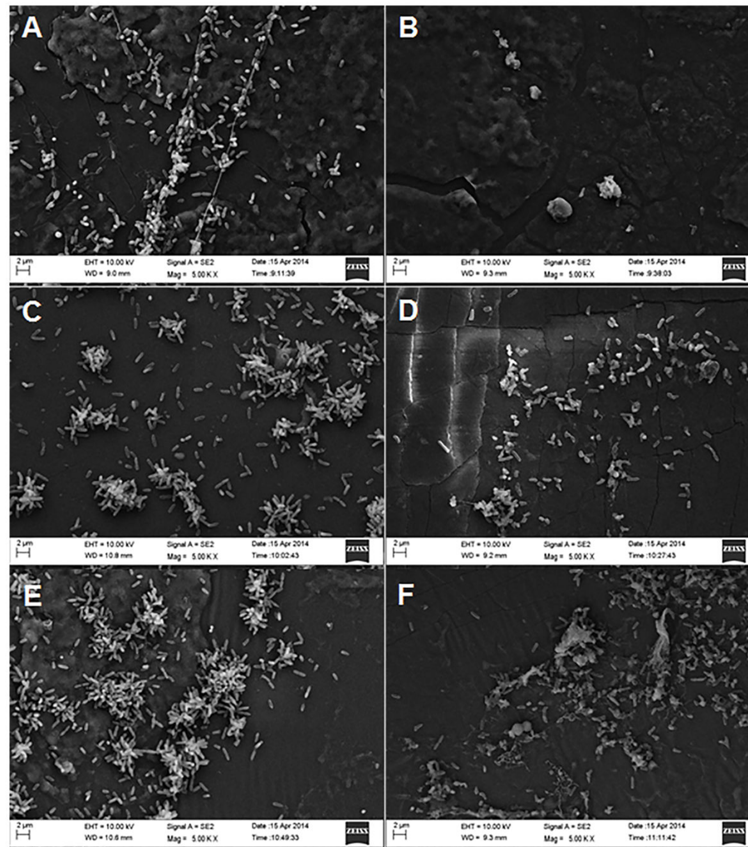

Figure 3. Scanning electron microscopy images of *P. aeruginosa* biofilms on the silicon backing of the wound dressing

Representative scanning electron microscopic images (5000X) of control *P. aeruginosa* biofilms (A, C, and E) and 10.0mM D-/L-tryptophan treated biofilms (B, D, and F) grown on the wound dressing for 24 h (A and B), 48 h (C and D), and 72 h (E and F). Images were taken with a LEO 1530 scanning electron microscope.

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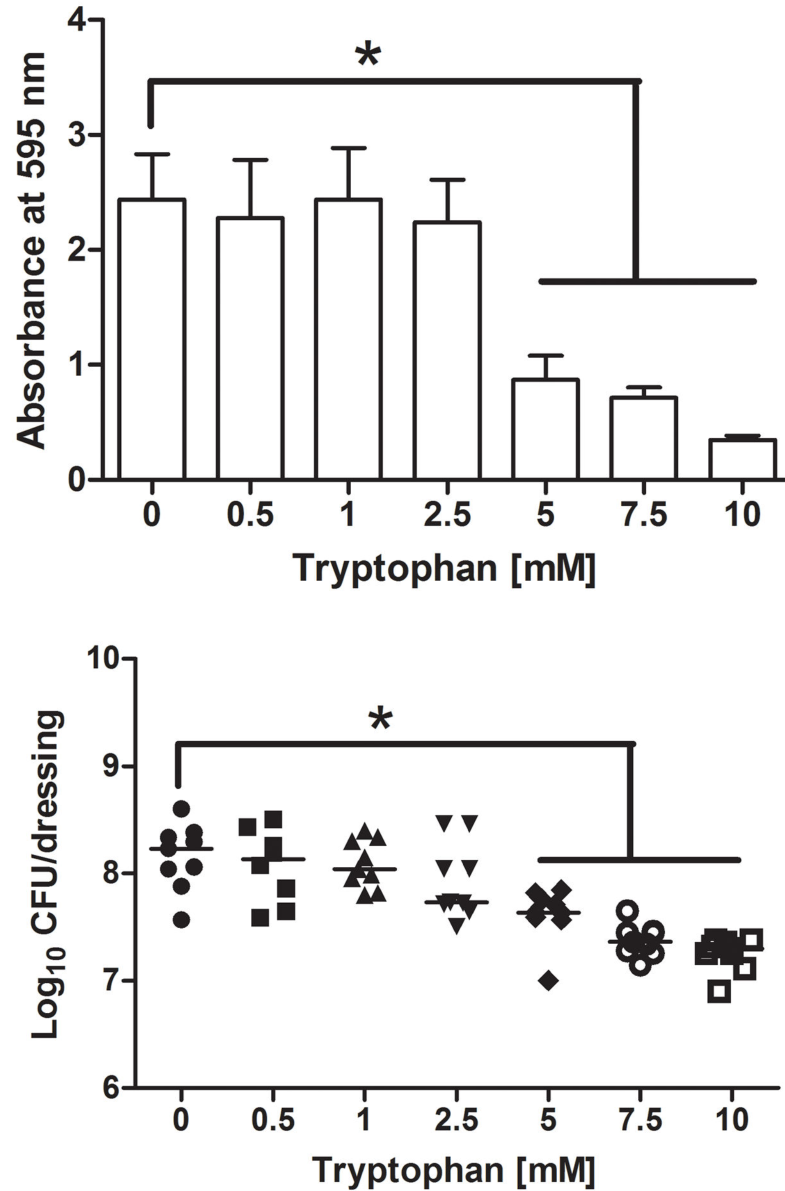

Figure 4. Dose-dependent inhibition of *P. aeruginosa* biofilms on a biological wound dressing by tryptophan

The bioloigcal wound dressing was bathed in *P.* aeruginosa suspended in M63 minimal media supplemented with tryptophan (0 – 10 mM) for 48 hours at 30°C. A) Tryptophan significantly inhibited biofilm formation on the dressing at concentrations above 5mM as determined by crystal violet staining (*, $p<0.05$). B) Tryptophan significantly decreased bacterial colonization of the dressing at concentrations above 5mM (*, $p<0.05$).

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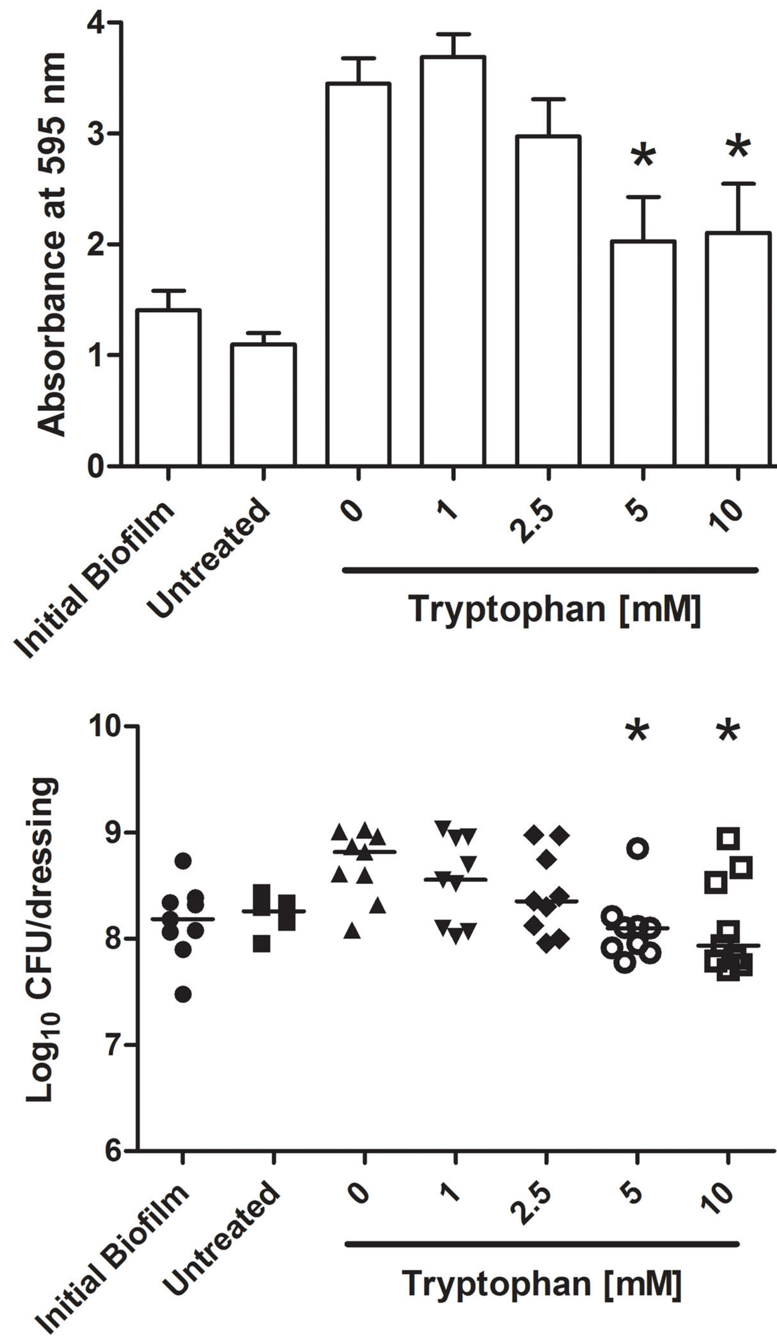

Figure 5. Tryptophan treatment of established *P. aeruginosa* biofilms

P. aeruginosa biofilms were grown for 48 hours at 30°C on the biological wound dressing prior to treatment with tryptophan (0 – 10 mM). The treatment lasted for an additional 24 hours at 30°C. A) Crystal violet staining revealed that tryptophan significantly inhibited additional biofilm growth (*, $p<0.05$) and that concentrations above 5mM were not significantly higher than the initial biofilm. B) Quantification of bacterial cells attached to the dressing showed that tryptophan significantly reduced additional bacterial colonization

compared to the fresh media alone (*, $p<0.05$), concentrations of tryptophan above 1mM were not significantly different than the initial bacterial load on the dressing.

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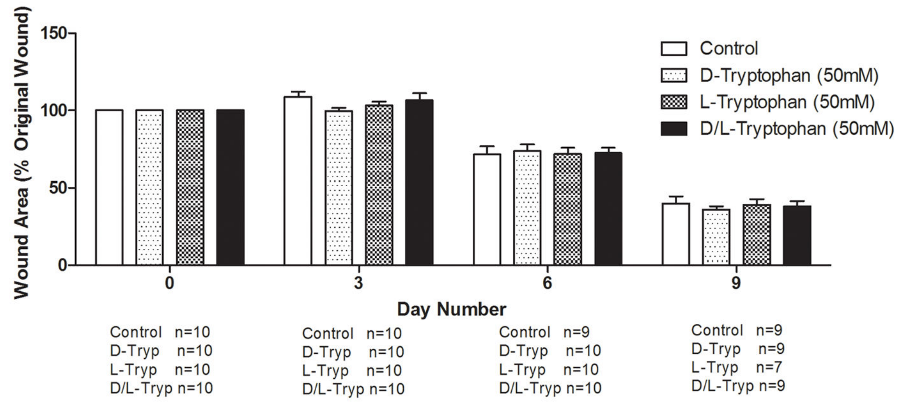

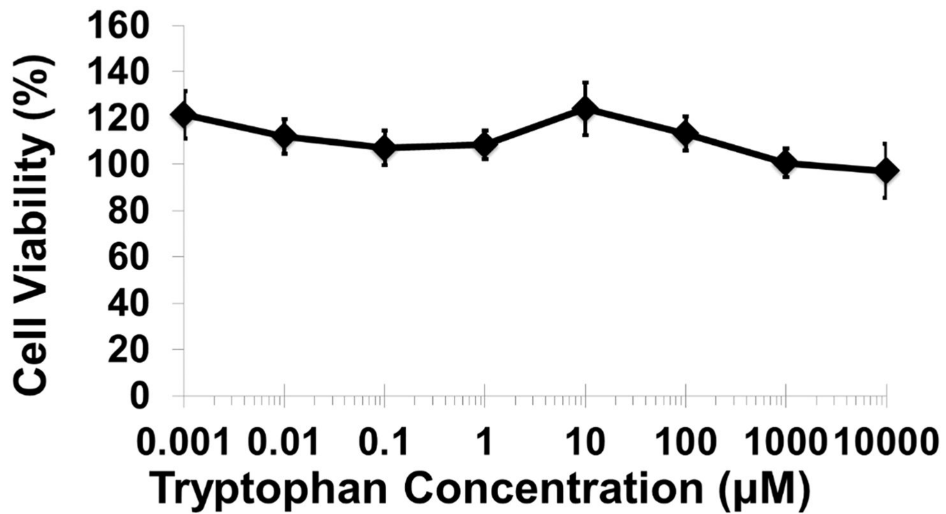

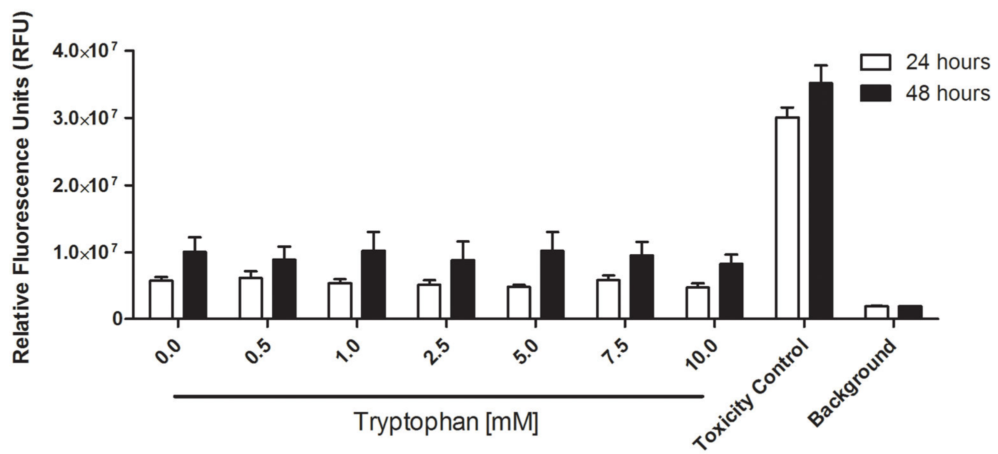

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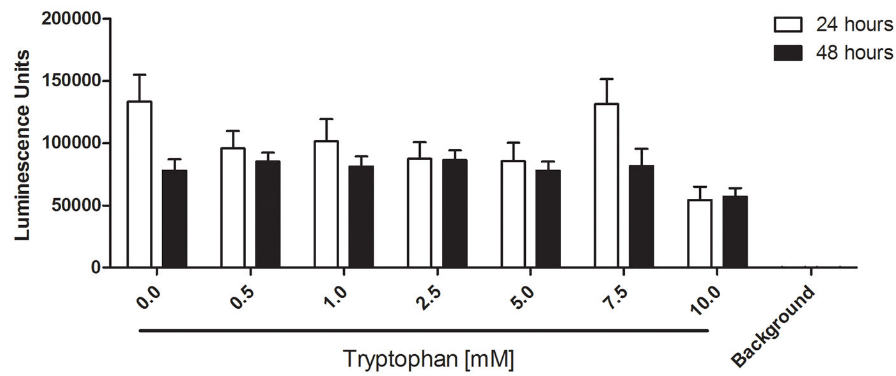

Figure 6. Tryptophan is not cytotoxic in murine skin wounds or for immortalized human keratinocytes

A) Twenty BALB/c mice were randomized to four treatment groups. Two splinted full thickness wounds were made on the backs of each mouse, and were treated with two 8mm diameter discs of Telfa® pads soaked with 60μl of either PBS (Control), 50mM D-tryptophan, 50mM L-tryptophan, or a 50:50 combination of D- and L-tryptophan (50mM total tryptophan concentration). Treatments were applied on day 0 and reapplied on days 3 and 6. Images of the wounds were taken on days 0 (baseline), 3, 6, and 9 with a Nikon D300 camera. Image J was used to calculate the wound areas, which were normalized to the baseline (day 0) values. No significant differences in remaining wound area were measured between any treatment and the control, a p-value of below 0.05 was considered significant. B) A one hour exposure of the immortalized HaCaT cell line to D-tryptophan at 37°C exhibited no direct cytotoxicity. Cell viability was measured using calcein-AM and measuring resulting fluorescence at EM:485nm/EX:528nm. C) No loss of cellular membrane integrity was observed in immortalized human keratinocytes (STINKs) with D-/L-tryptophan concentrations up to 10mM over 24 and 48 hour incubations at 37°C using the CellTox™ Green Cytotoxicity Assay. D) Altered or reduced the cellular metabolism of the STINKs cell line was observed over 24 and 48 hour incubations with increasing D-/L-tryptophan concentrations as assessed by the RealTime-Glo MT Cell Viability Assay.

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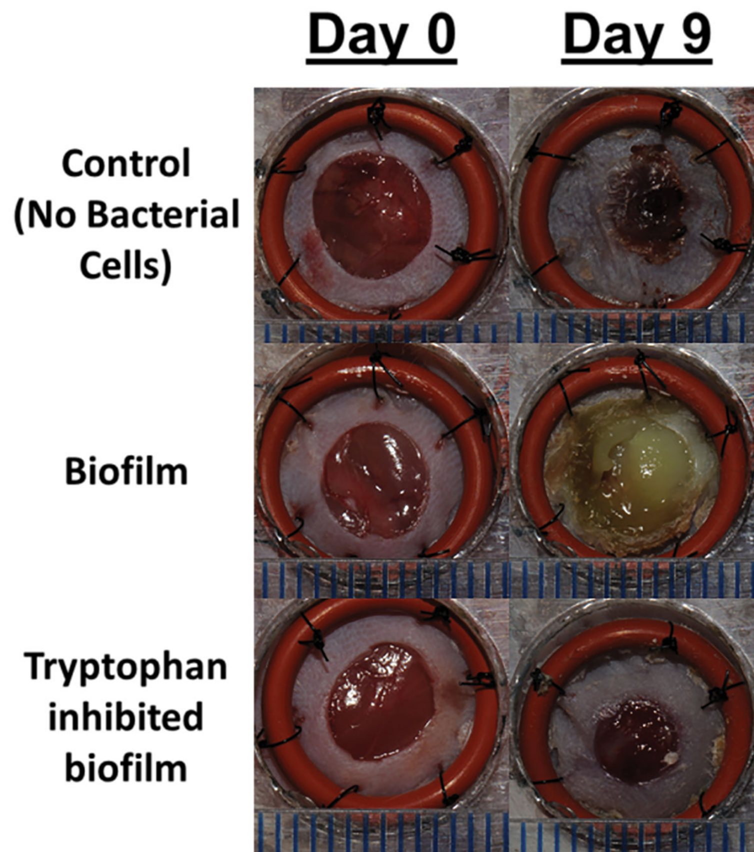

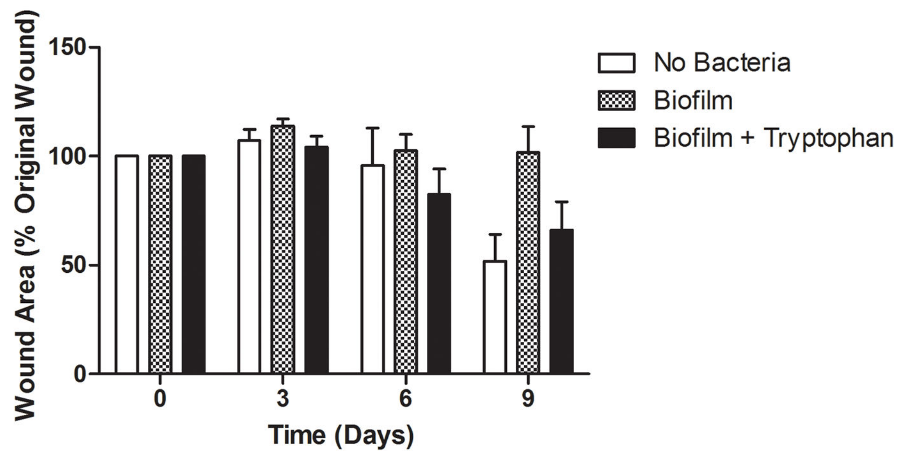

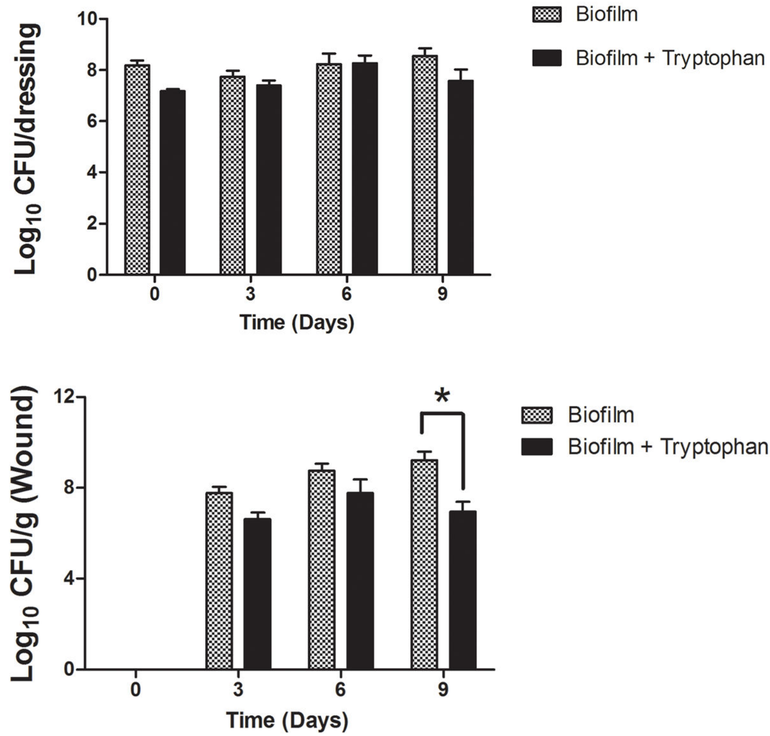

Figure 7. *P. aeruginosa* biofilms on wound dressings delay wound healing

P. aeruginosa biofilms with or without 10mM D-/L-tryptophan were established on wound dressings for 48 hours at 30°C. The contaminated dressings were applied to mouse skin wounds for up to 9 days. On days 3, 6, and 9, two mice from each group were sacrificed for imaging of the wounds, bacterial quantification of the dressing and wound bed. A) Representative images of the wounds in the three groups on days 0 and 9. B) Wound areas are expressed as percentages of the day 0 baseline (100%). By day 9 the control wounds without *P. aeruginosa* were ~50% closed, the biofilms grown in the presence of tryptophan were ~35% closed, and the biofilms grown without tryptophan remained fully open. C) Bacterial loads on the dressing ($\log_{10}$ CFU/dressing) did not change significantly over the 9 days on the mouse skin wounds. D) Tryptophan slightly reduced the bacterial loads on the wound bed ($\log_{10}$ CFU/g) with significantly lower counts detectible on day 9.

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