Effect of Proteasome Inhibitor 1 on Wound Healing: A Potential Scar Prevention Therapy

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Abstract: In vitro and in vivo assessments suggest that proteasome inhibitors may be useful for modulating wound healing. Methods. Proteasome Inhibitor I was used to assess the potential utility of proteasome inhibitors in improving wound healing in a standard rat model. Bilateral, 6 cm incisions were made 1 cm lateral to the spine of adult male Sprague Dawley rats. Animals were randomly assigned to 1 of 3 groups: no treatment (n = 15), low concentration (1% w/v, n = 15), or high concentration (5% w/v, n = 15). Treatments were applied to the left side incision at 0 hours, 24 hours, and 48 hours. Right-side incisions received a vehicle, dimethyl sulfoxide, alone and independent of the assigned group, serving as both external and internal controls. Rats were sacrificed at days 7, 14, and 28 (n = 5 per group) and wounds subjected to mechanical testing and histology. Results. No significant intergroup difference existed at 7 and 14 days. On day 28, a dose-dependent increase in tensile strength with increasing Proteasome Inhibitor I was observed. Conclusion. Results suggest dimethyl sulfoxide was not the ideal vehicle and additional improvement may be realized by optimizing the delivery method.

Abnormal scarring is a source of significant morbidity, and thus, is an active area of research. Much of this research is focused on elucidating the normal and pathological processes that differentiate acceptable scars from those that are either excessive or suboptimal. A significant insight from this body of work is the importance of inflammation in the progression of wound healing, with such insights having the potential to lead to innovative treatments. Important findings in scarless healing of fetal skin include an altered expression of matrix metalloproteases (MMPs), decreased expression of proinflammatory cytokines, and healing in the presence of reduced inflammation. The role of TGF-β in scarring has been extensively studied with evidence supporting that high levels of isoforms 1 and 2 play key roles in the scarring process. However, no pharmaceutical therapy exists for scar prevention, because those currently available either lack sound evidence of benefit, have limited applications, or possess detrimental side effects.

Recently, proteasome inhibitors (PI) have come under closer investiga-
tion as potential antifibrotic therapeutics. Proteasome inhibitors have shown promise as potential anti-fibrotic agents in multiple fibrotic models including renal fibrosis, cardiac fibrosis, myelodysplasia, pulmonary fibrosis, and skin fibrosis.\textsuperscript{12-15} Proteasome inhibitors exert direct cellular effect via inhibition of the 20S proteasome, a barrel-structured cytosolic enzyme that degrades intracellular proteins tagged for destruction through the ubiquitin pathway. Recent \textit{in vitro} and \textit{in vivo} work demonstrate PI can downregulate the inflammatory response through inactivation of nuclear factor (NF)-kB; block antifibrotic effects associated with TGF-\textbeta; alter MMP and TIMP expressions; and decrease collagen 1 synthesis.\textsuperscript{12,16} While most of these cellular effects lack well-delineated pathways and warrant further interrogation, they reflect changes that parallel fetal scarless healing over scarring.

Proteasome inhibitors have not been explored in wound healing as a potential scar preventive therapy. Thus, the purpose of this study was to qualify proteasome inhibitor 1 (PI1), an aldehyde peptide, for further investigation in a future scar model by first investigating its effects on acute wound healing and further interrogating cellular effects. The authors hypothesized that wounds treated with PI1 would heal with a dermal architecture closer to that of normal skin when compared to controls, thus providing a wound with superior mechanical integrity. To test this hypothesis, a standard rat incisional wound model with a primary end point of mechanical wound strength was used.

**Key Points**

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**Methods**

All animal surgery was performed following Institutional Animal Care and Use Committee-approved protocol. As described by Mustoe et al.,\textsuperscript{17} two 6 cm linear incisions were made through the panniculus carnosum on the dorsal aspect of 10-12 week-old male Sprague Dawley rats (Harlan, Houston, TX, USA) with 1 cm on either side of midline running cephalid to caudid. A prefabricated template was used to ensure incisions were placed in a consistent location on each rat. Three surgical clips were used to close each incision and serve as guides for sectioning on harvest day.

A total of 45 rats were randomized to 3 treatment groups. All wounds located on the right received vehicle alone—100% dimethyl sulfoxide (DMSO)—serving as both an external control and an internal control, to control for both DMSO and systemic effects. Group 1 remained untreated; Group 2 received PI1 (Calbiochem, San Diego, CA, USA) at 1% w/w in DMSO; Group 3 received PI1 at 5% w/w in DMSO (Figure 1). Treatment was administered by applying 100 \muL to each wound every 24 hours for the first 48 hours, beginning at the time of wound closure, for a total of 3 doses per wound. The wounds were left uncovered and assessed twice daily. No evidence of the rats disturbing the wounds was apparent.

On days 7, 14, and 28 post-wounding, 5 animals from each group were euthanized and the entire dorsum excised. Four 8 mm sample strips were cut perpendicular to the wounds, incorporating both the PI1-treated wound and the adjacent vehicle-treated wound (internal control). These strips were then cut in half, equidistant from the 2 parallel wounds, providing an anatomically matched complimentary vehicle-treated wound for each of the experimentally treated wound samples. One cephalic and 1 caudal sample were immediately prepared for histology, with the remaining 2 strips immediately prepared for tensometry.

**Tensometry.** Prior to sample loading, the underlying
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Subcutaneous fascia was removed to the level of the panniculus carnosum using sharp dissection, along a natural dissection plane. For the day 28 wounds, the 8 mm samples were further divided into 4 mm strips prior to loading. Samples were loaded into a Lloyd Instrument materials testing system (LRX plus, Fareham, Hampshire, UK) with a 50 N load cell, with the wounds perpendicular to the grips and pulled at a rate of 200 mm/min. Wound thickness, superficial to the epithelium and as deep as the panniculus carnosum, was measured at the time of testing with digital calipers (Absolute Digimatic Caliper, Mitutoyo, Kawasaki, Kanagawa, Japan). The wound width was measured at a preload of 0.5 N by obtaining a time-correlated still frame image from a mounted video camera (HDR-SR1 Handycam® camcorder, Sony, San Diego, CA, USA). These 2 measurements were then used to calculate the cross sectional area of the wounds and the subsequent tensile strength (N/mm²).

Histology. Samples were fixed in 10% formalin, embedded in paraffin, and sectioned at approximately 5 μm. Cross sections of skin were stained with hematoxylin and eosin (H&E) and Masson’s Trichrome. All slides were subjectively evaluated on an Olympus BX40 microscope (Olympus America, Center Valley, PA, USA) by a board-certified veterinary pathologist blinded to the treatment groups. The following parameters were determined for each sample: (1) collagen density (0 = no mature collagen, 1 = loose, 2 = intermediate, 3 = dense); (2) collagen maturity (1 = immature, 2 = intermediate, 3 = mature); (3) amount and type of inflammation present in the dermis (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe); and (4) epithelial hyperplasia (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe). Collagen density and maturity scores were then averaged to provide an overall collagen score. Images were captured using an Olympus BX41 microscope (Olympus America, Center Valley, PA) and an Olympus DP71 digital camera (Olympus America, Center Valley, PA, USA).

Statistics Analysis

Statistical analysis was performed using STATA (StataCorp, College Station, TX, US) with analysis of the variance used to determine significant differences among treatment groups with multiple comparisons adjusted for using Bonferroni’s correction. A paired Student’s t test was used to determine significant differences between treated wounds and internal controls. A Kruskal-Wallis test was used to determine significant differences for all graded histology. Significance was set to \( P < 0.05 \).

Results

Tensile strength increased with time post-wounding in all groups (Figure 2). No significant differences in tensile strength existed between treatment groups at days 7 and 14 post-wounding. On day 28, a dose-dependent effect existed, with wounds receiving 5% PI1 having significantly increased tensile strength over Group 1 vehicle control wounds (\( P = 0.04 \) (Figure 3). Tensile strength of Group 2 (1% PI1) did not differ significantly from that of Group 3 (5% PI1) or from the vehicle control. Intra-rat wound comparison with contralateral vehicle controls failed to demonstrate a significant difference within all 3 groups with the exception of day 14, when 5% PI1-treated wounds which had inferior strength to contralateral vehicle controls, 0.46 versus 0.77 N/mm² (\( P = 0.002 \).

Wound thickness, as measured from epithelium to just beneath the panniculus carnosum, decreased over the healing course for all groups. On day 28, a dose-dependent decrease in wound thickness was observed with 5% PI1 wounds being statistically thinner than Group 1 vehicle control wounds, 1.79 mm versus 2.17 mm (Figure 4).

Histology revealed time-dependent changes of dermal healing, beginning with granulation tissue seen on day 7, followed by primarily disorganized collagen on day 14, and ending on day 28 with basket weaved collagen consisting of collagen fibrils with decreased diameters compared to adjacent non-wounded skin. Graded histology revealed no statistical differences in the collagen progression between control, vehicle, or treated wounds.

Inflammatory response, as measured by cellular infiltration of the dermis by neutrophils (acute), macrophages, and lymphocytes (chronic), presented with pronounced day 7 infiltrates with an expected time-dependent decline (\( P < 0.05 \)). Epidermal hyperplasia was pronounced on day 7, followed by a decrease over time (\( P < 0.05 \). No statistical difference existed between groups for any time period evaluated.

Finally, histology revealed several PI1-treated wounds...
to contain a foreign body reaction in the subcutaneous tissues, just beneath the panniculus carnosum. The number and size of the reactions had a subjective dependence upon the dose received. None of the internal controls demonstrated this reaction.

**Discussion**

Given the prevalence of wounds and their frequent pathological outcomes, development of a pharmaceutical for scar prevention is poised for widespread impact. This study provides preliminary evidence supporting therapeutic potential with the local administration of PI1. The results demonstrate improved wound healing with local administration of PI1 as evidenced by a 44% and 25% gain in tensile strength among wounds locally treated with 5% PI1 and 1% PI1, respectively, compared to Group 1 vehicle control wounds at day 28. Furthermore, macroscopic evaluation revealed thinner wounds with histological correlates occasionally demonstrating wound architecture that was more reflective of unwounded skin, which suggested an anti-scarring effect was present.

Improved outcomes within this study are likely partially attributed to PI1’s anti-inflammatory properties. Histological evaluation of wounds revealed an expected decrease in inflammatory response among treated wounds first characterized by diminished neutrophil infiltrates on day 14, most notable among the 1% PI1 treatment group ($P = 0.12$). This anti-inflammatory effect was further demonstrated on day 28 with PI1 treated wounds having a decreased accumulation of macrophage and lymphocytic infiltrates, again most notably in the 1% PI1 rat group ($P = 0.11$). This finding is consistent with *in vitro* and *in vivo* work demonstrating the ability of PI1 to block the inflammatory response through the NF-κB and TGF-β pathways. While early literature urges against the use of preventive anti-inflammatory therapy secondary to failure to heal, recent reports suggest immune modulation may optimize wound healing. Recent studies investigating anti-TGF-β and selective COX-2 inhibitors show wound healing occurs in the face of a decreased immune response with less collagen deposition observed, while maintaining wound strength. This study, in congruence with recent reports, supports the idea that early intervention in wound healing provides late beneficial effects but also reinforces use of caution since a delay to improved strength was also observed. This conclusion, however, mandates further investigation, since the delivery vehicle, DMSO, exerts anti-inflammatory effects that confounded interpretation.

![Figure 2. Gain in tensile strength over time for treated wounds. (n = 5, *P < 0.05)](image)

![Figure 3. Tensile strength on day 28 post-wounding, demonstrating dose-dependent gain in tensile strength. (n = 5, *P < 0.05)](image)

![Figure 4. Thickness of healing skin. (n = 5, *P = 0.05)](image)
Interestingly, an anti-inflammatory effect may not be solely responsible for improved outcomes. The Group 1 vehicle DMSO control wounds, also showed a decreased day-14 inflammatory response \((P = 0.20)\), compared to nontreated controls, and subsequently failed to improve day-28 strength outcomes. Additionally, both the 5% PI1 and its contralateral vehicle control wounds lacked the same diminished day-14 cellular infiltrate observed in the 1% PI1-treated wounds. Given these observations, we conclude that while the anti-inflammatory component plays a role, another mechanism is in part responsible for the improved outcomes.

The authors observed the hastened epidermal healing among the PI1-treated wounds might, in part, be responsible. Pathology-graded epidermal hyperplasia was used to gauge epidermal maturation with nearly all PI1-treated wounds to include contralateral vehicle control wounds reflecting a normal appearance by day 28. This finding was independent of DMSO effects and is consistent with ex vivo data from the authors’ lab showing PI1 administration to human skin equivalents initiates epidermal changes, according to oral communication and email correspondence with Gianni Rossini, MS, senior research scientist at the Southwest Research Institute (San Antonio, TX). Other investigators have shown that epidermal-dermal cross talk is vital to wound maturation, with one study demonstrating that the source of keratinocytes can affect the dermal outcome in skin equivalents.\(^{25,26}\) Since the less mature-appearing collagen typically occurred in the superficial third to half of the dermis when present, the authors speculate the epidermal findings play a role in collagen maturity.

While a late benefit was achieved, early wound healing effects were unclear secondary to confounding deleterious effects of the delivery vehicle. Relative to the untreated wounds, all other wounds demonstrated statistically significant decreased tensile strength on day 7, with PI1-treated wounds having comparable strength to both their contralateral vehicle and Group 1 vehicle control wounds, indicating the adverse effects of DMSO masked any early beneficial effects of PI1. Therefore, further investigation in which a different vehicle or route of administration are used may shed light on these findings.

Since PI1-treated wounds tend to present with granulomatous reactions deep to the dermis, refinement of delivery method and dose is required. A delivery vehicle, such as a topical ointment, may prevent precipitation of the active agent. While dimethyl sulfoxide is a common medium for improving transdermal delivery of pharmaceutical compounds and an appropriate choice for intact skin in this case, the authors believe the delivery of the DMSO/PI1 solute deep in the wound bed resulted in rapid DMSO absorption leading to precipitation of PI1. Such precipitate may explain the counterproductive foreign body reactions observed. This mechanism was proposed after observing quick absorption of the DMSO on the intact wounds with white precipitate remaining.

**Conclusion**

The data supports further work to determine if PI1 is a candidate as a pharmaceutical therapy to improve acute wound healing, despite the questionable effects of this study’s chosen delivery method and vehicle. Furthermore, it is clear that early administration of PI1 modifies the wound environment so that effects are propagated well beyond the initial days of administration. Proteasome inhibitor 1 also appears to redirect the healing process toward regeneration with an end product suggestive of reduced scarring with improved wound strength. While this study’s results are promising, there remains a need for future investigations to further ascertain whether the local application of PI1 exerts systemic effects, along with verifying its ability to prevent scarring in a formal scar model.

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References


