Acceleration of diabetic wound healing using a novel protease–anti-protease combination therapy

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Nonhealing chronic wounds are major complications of diabetes resulting in >70,000 annual lower-limb amputations in the United States alone. The reasons the diabetic wound is recalcitrant to healing are not fully understood, and there are limited therapeutic agents that could accelerate or facilitate its repair. We previously identified two active forms of matrix metalloproteinases (MMPs), MMP-8 and MMP-9, in the wounds of *db/db* mice. We argued that the former might play a role in the body's response to wound healing and that the latter is the pathological consequence of the disease with detrimental effects. Here we demonstrate that the use of compound ND-336, a novel highly selective inhibitor of gelatinases (MMP-2 and MMP-9) and MMP-14, accelerates diabetic wound healing by lowering inflammation and by enhancing angiogenesis and re-epithelialization of the wound, thereby reversing the pathological condition. The detrimental role of MMP-9 in the pathology of diabetic wounds was confirmed further by the study of diabetic MMP-9–knockout mice, which exhibited wounds more prone to healing. Furthermore, topical administration of active recombinant MMP-8 also accelerated diabetic wound healing as a consequence of complete re-epithelialization, diminished inflammation, and enhanced angiogenesis. The combined topical application of ND-336 (a small molecule) and the active recombinant MMP-8 (an enzyme) enhanced healing even more, in a strategy that holds considerable promise in healing of diabetic wounds.

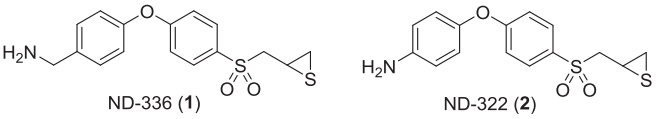
diabetic wound healing | MMP-8 | MMP-9 | inhibition | ND-336

Diabetes affects 340 million people in the world, including 29.1 million individuals in the United States (1). A complication in diabetic patients is the inability of wounds to heal, which resulted in 73,000 lower-limb amputations in the United States in 2010 (1). The standard treatment for diabetic foot ulcers includes debridement of the wound, treatment of infection with antibiotics, and reducing or eliminating weight pressure from the lower extremities (2). There is a paucity of pharmacological therapeutics that accelerate wound healing. Although becaplermin (PDGF) is approved for use in diabetic neuropathic ulcers, malignancies have been reported, and an increased risk of mortality was observed in patients treated with becaplermin (3).

In diabetic patients, high blood sugar triggers prolonged chronic inflammation, with concomitant elevated levels of matrix metalloproteinases (MMPs). The detrimental effect of MMPs in the diseased tissue has been attributed to the rapid turnover of potential growth factors, receptors, and the newly formed extracellular matrix, which are essential for wound healing (4). Hence, wound healing is impaired and delayed in diabetic patients. However, this process is not well understood, and the actual instigator MMPs are not known.

MMPs are a family of zinc-dependent endopeptidases that are capable of degrading extracellular matrix components and are involved in tissue remodeling and restructuring (5). MMPs are expressed as zymogens or pro-MMPs. Activation by proteolytic removal of the N-terminal prodomain is required for their catalytic functions. Active forms of MMPs are highly regulated by binding of tissue inhibitors of metalloproteinases (TIMPs). MMPs are presumed to play various roles in regulating inflammatory and repair processes (6) as well as in wound healing (7).

We recently reported on the identification and quantification of active MMP-8 and MMP-9 in a mouse model of diabetic wound healing by the use of an inhibitor-tethered resin that binds exclusively to active MMPs, in conjunction with proteomics analyses (8). Because MMP-9 was observed to be up-regulated only in diabetic wounds, whereas MMP-8 was found in both diabetic and nondiabetic wounds, we hypothesized that MMP-8 is beneficial and that MMP-9 is detrimental in diabetic wound healing. We now report that the use of either a novel and highly selective MMP-9 inhibitor of our design (ND-336, compound **1**) or the application of the active recombinant MMP-8 accelerates wound healing in *db*/*db* mice. We further confirm the detrimental effect of MMP-9 on diabetic wound healing by the use of MMP-9–knockout mice. Finally, we document that the combination of a selective MMP-9 inhibitor (a small molecule) plus the active recombinant MMP-8 (an enzyme) accelerated wound healing even further in *db*/*db* mice. This combination is a potential pharmacological treatment for diabetic wound healing and holds great promise for recourse in this devastating disease.



Scheme 1. Structures of compounds **1** and **2**.

Results and Discussion

Synthesis and MMP Inhibition Profile of ND-336. There are 23 MMPs in humans (5), and their catalytic domains are very similar in structure; thus, the design of inhibitors that are selective for a particular MMP is extremely challenging. In fact, most inhibitors of MMPs are zinc chelators, which broadly inhibit many or all MMPs (9) and the related ADAMs (a disintegrin and metalloproteinase).

Significance

Chronic wounds in diabetic patients are a devastating complication of diabetes that can lead to amputations or even death. Our work in *db/db* mice shows that matrix metalloproteinase (MMP)-9 contributes to delayed or impaired wound healing and that MMP-8 is involved in repairing the wound. A combination of a selective inhibitor of MMP-9 (a small molecule) and exogenously applied active recombinant MMP-8 (an enzyme) accelerates diabetic wound healing in mice.

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The authors declare no conflict of interest.

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Over the past several years we have produced a library of thiirane inhibitors for MMPs (10–12), which allowed the identification of specific inhibitors for selective inhibition of enzymes involved in various MMP-mediated diseases. The thiiranes undergo a reaction catalyzed by the target MMP, resulting in opening of the thiirane ring and generation of the thiolate, which is a tight-binding inhibitor (13). When we identified active MMP-8 and MMP-9 in the diabetic wounds and hypothesized that MMP-9 plays a detrimental role in the disease, the central criterion for selectivity of a suitable inhibitor became its ability to differentiate between MMP-8 and MMP-9, because the latter had to be inhibited in the presence of active MMP-8. We now report on the discovery of compound **1** (as shown in Fig. S1), which meets the requirement for potent inhibition of MMP-9 and lack thereof for MMP-8.

The binding constants for ND-336 with seven representative MMPs and two related ADAMs are given in Table 1. ND-336 inhibits MMP-2, MMP-9, and MMP-14 in a slow-binding mechanism, with inhibition constant (K_i) values of 85 ± 1 nM, 150 ± 10 nM, and 120 ± 10 nM, respectively. Because MMP-2 and MMP-14 are absent in the diabetic wound (8), the inhibitor essentially targets MMP-9 in this microenvironment. The residence times (the time the drug remains bound to the target; calculated as $1/k_{off}$) (14) of ND-336 are 23.4 ± 0.2 min for MMP-2, 47.4 ± 4.4 min for MMP-9, and 12.6 ± 0.3 min for MMP-14. For comparison, the residence times of the endogenous protein inhibitors TIMPs are shorter: 6.9 min for MMP-2-TIMP1, 10.4 min for MMP-2-TIMP2, 7.9 min for MMP-9-TIMP1, and 6.7 min for MMP-9-TIMP2 (15). That is, ND-336 is better at inhibiting MMP-2 and MMP-9 than are the TIMPs that have evolved for this purpose. ND-336 exhibits marginal to no inhibition of MMP-1, MMP-3, MMP-7, ADAM9, and ADAM10, and it poorly inhibits MMP-8 in a linear noncompetitive manner (K_i = 7,700 ± 100 nM). Combined with the 50-fold lower K_i for MMP-9, the exceptional residence time of ND-336 contributes substantially to its selectivity. The residence time is an important contributor to the effective inhibition of MMP-9, in contrast to the linear noncompetitive inhibition of MMP-8 by ND-336, with a very short residence time and poorer dissociation constant.

Inhibition of MMP-9 with ND-336 in Diabetic Wound Healing. ND-336 was evaluated in a mouse model of diabetic wound healing. Excisional wounds were made on the dorsal thorax of *db*/*db* mice, and the wounds were treated topically with vehicle, ND-336, or ND-322 (compound **2**), which was used as a positive control. ND-322 inhibits MMP-9 as a slow-binding inhibitor with a K_i of 870 ± 110 nM and inhibits MMP-8 as a linear noncompetitive inhibitor with a K_i of 2,600 ± 400 nM, with a threefold selectivity for MMP-9 over MMP-8 (10). Wounds treated with ND-336 healed 1.2- to 1.6-fold faster than those treated with ND-322 and twofold faster than those treated with vehicle (Fig. 1*A*). We attribute the superior efficacy of ND-336 over ND-322 to ND-336's more selective inhibition of MMP-9 than of MMP-8. Because human wounds heal by re-epithelialization, we evaluated the wounds with H&E staining to visualize the epithelium. Treatment with ND-336 resulted in almost complete re-epithelialization compared with partial re-epithelialization in the ND-322– and vehicle-treated groups (Fig. S2*A*). Because MMP-9 activity is associated with the induction of apoptosis (16), we evaluated the wounds by the TUNEL, which detects DNA fragmentation resulting from apoptotic cells. As shown in Fig. S2*B*, numerous apoptotic cells were found in the vehicle-treated group, but apoptosis was significantly decreased in the ND-322– and ND-336–treated groups. In situ zymography detects MMP activity in vivo using the fluorogenic substrates DQ-gelatin for gelatinase (MMP-2 and MMP-9) activity and DQ-collagen for collagenase (MMP-1, MMP-8, and MMP-13) activity. Because only active MMP-8 and MMP-9 were identified by our proteomics analyses in *db*/*db* wounds (8), the gelatinase activity observed by in situ zymography corresponds to MMP-9 activity, and the collagenase activity is reflective of MMP-8 activity. Treatment with ND-336 significantly decreased MMP-9 activity (Fig. S2*C*, *Bottom Left*), but MMP-8 activity was not affected (Fig. S2*D*, *Bottom Left*), as expected from the kinetic profile of ND-336 (Table 1). In contrast, treatment with ND-322 significantly decreased MMP-9 activity (Fig. S2*C*, *Middle Left*); however, it also decreased MMP-8 activity (Fig. S2*D*, *Middle Left*). Merged images stained with DAPI (blue) indicated comparable numbers of nuclei in the wound tissues treated with vehicle, ND-322, and ND-336 (right panels in Fig. S2 *C* and *D*).

Ablation of MMP-9 in Diabetic Wound Healing. We induced diabetes in MMP-9–knockout mice to confirm the detrimental role of MMP-9 in diabetic wound healing. We administered streptozotocin, which destroys insulin-producing beta cells in the pancreas by necrosis (17) and used wild-type mice treated with streptozotocin as the control group. As shown in Fig. S3, the wounds of streptozotocin-treated MMP-9–knockout mice healed faster than those of streptozotocin-treated wild-type mice and resulted in complete re-epithelialization and in diminished apoptosis. This study confirmed that MMP-9 is involved in the pathology of diabetic wounds. Because enhanced expression of MMP-8 has been reported in MMP-9–knockout mice (18), the acceleration of wound healing that we observe in diabetic MMP-9–knockout mice could be explained by the up-regulation of MMP-8 and the absence of MMP-9. Our findings in diabetic MMP-9–knockout mice differ from those of Kyriakides et al. (19), who suggested that MMP-9 is required for normal progression of wound closure because MMP-9 gene ablation in nondiabetic mice led to delayed wound healing caused by compromised re-epithelialization, attenuated keratinocyte wound migration, and reduced clearance of fibrin clots. However, inflammation and angiogenesis in wounds of nondiabetic MMP-9–knockout mice were similar to those in control mice (19). Others have found that skin inflammation is alleviated in MMP-9–knockout mice (20) and that inhibition of MMP-9 with leptomycin B suppressed inflammation in UVB-irradiated murine skin (21), consistent with our findings.

Effect of MMP-8 in Diabetic Wound Healing. To test the hypothesis that MMP-8 contributes to the repair in diabetic wound healing, we evaluated the effect of topical application of the protease MMP-8

Table 1. Inhibition profile of ND-336

Enzyme	Inhibition type	k_{on}, $s^{-1}M^{-1}$	k_{off}, $10^3 s^{-1}$	K_i
MMP-1*				4% inhibition @ 100 μM
MMP-2	Slow-binding	8,380 ± 110	0.712 ± 0.006	85 ± 1 nM†
MMP-3*				23% inhibition @ 100 μM
MMP-7				1% inhibition @ 100 μM
MMP-8*	Linear noncompetitive			7,700 ± 100 nM
MMP-9*	Slow-binding	2,360 ± 100	0.352 ± 0.033	150 ± 10 nM†
MMP-14*	Slow-binding	10,800 ± 400	1.33 ± 0.03	120 ± 10 nM†
ADAM9				31% inhibition @ 100 μM
ADAM10				14% inhibition @ 100 μM

*Catalytic domains.
†Calculated from the ratio of k_{off}/k_{on}.

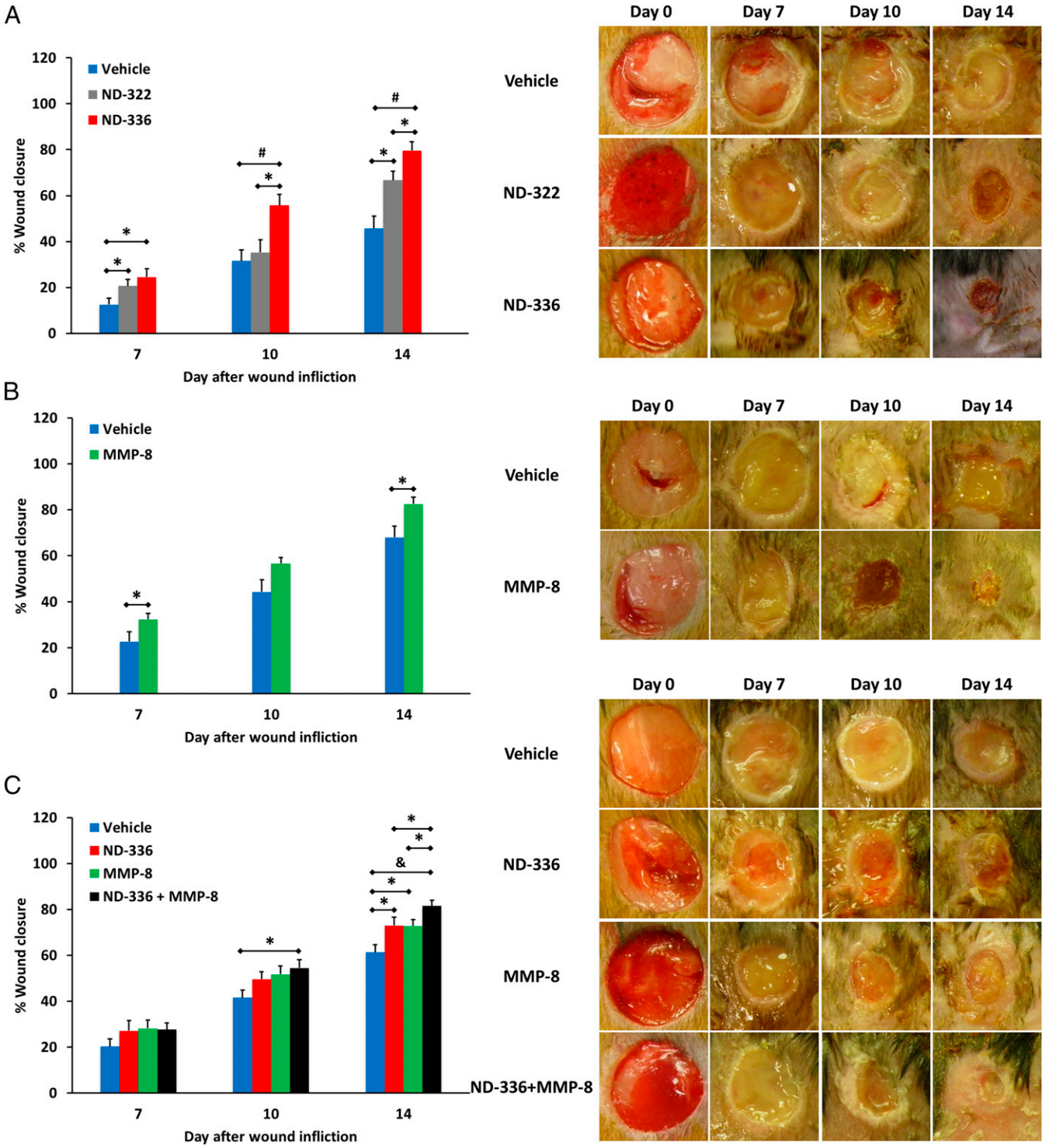

Fig. 1. Effect of MMP-9 inhibition, topical treatment with exogenously added MMP-8, and combined MMP-9 inhibition and exogenous MMP-8 on diabetic wound healing. A single 8-mm wound was made on the dorsal thorax of *db*/*db* mice. $^{*}P < 0.05$, $^{\#}P < 0.01$, $^{\&}P < 0.001$ indicate statistically significant differences in wound closure between the indicated groups. Statistical significance was evaluated by the two-tailed Mann–Whitney *u* test. (*A*) Wound healing after treatment with ND-336 (0.1 mg per wound per day), ND-322 (0.1 mg per wound per day) as a positive control, or vehicle. Data are shown as mean ± SEM ($n = 8$ mice per group on days 7, 10, and 14). (*B*) Wound healing after exogenously added MMP-8 (1 μg per wound per day). Data are shown as mean ± SEM ($n = 20$, 9, and 9 mice on days 7, 10, and 14, respectively, for the vehicle group; $n = 20$, 10, and 10 mice on days 7, 10, and 14, respectively, for the MMP-8 group). (*C*) Wound healing after treatment with combined ND-336 (0.05 mg per wound per day) and MMP-8 (1 μg per wound per day). Data are shown as mean ± SEM; $n = 13$, 14, 12, and 12 for the groups treated with vehicle, ND-336, MMP-8, and ND-336 + MMP-8, respectively.

to wounds of *db*/*db* mice. We cloned the gene and expressed and purified active murine recombinant MMP-8 (*SI Materials and Methods*). The active recombinant MMP-8 was applied topically to the wounds at 10-fold the level found in the wounds (8). MMP-8 accelerated wound healing at this level in *db*/*db* mice, with statistical differences observed on days 7 and 14 (Fig. 1*B*),

and resulted in complete re-epithelialization (Fig. S4*A*). Increased MMP-8 activity in the MMP-8–treated *db*/*db* mice was confirmed by in situ zymography (Fig. S4*B*). This study indicated that MMP-8 plays a beneficial repair role in diabetic wound healing. Our findings are in agreement with those of Gutiérrez-Fernández et al. (22), who found that nondiabetic mice deficient in MMP-8 have delayed wound healing. Interestingly, MMP-8–knockout mice have significantly increased levels of MMP-9 (22), because of compensatory expression, which contributes to delayed wound healing.

Effect of the Combination of MMP-9 Inhibitor and Exogenously Added Active Recombinant MMP-8. Because either inhibition of MMP-9 by ND-336 or topically applied exogenously added active recombinant MMP-8 alone accelerated wound healing, we investigated the effect of the combination therapy. We first determined that the application of 0.05 mg ND-336 per wound per day was the lowest dose that accelerated wound healing in *db*/*db* wounds (Fig. S5). We also determined that 1 μg of MMP-8 per wound per day was the dose that most effectively accelerated wound repair (Fig. S6). As seen in Fig. 1*C*, the combined treatment not only showed significant acceleration of wound healing compared with the vehicle group on both days 10 and 14, but also showed significantly faster healing on day 14 than when a single agent was used in the treatment. Histological assessment of the wounds revealed that the combination of the MMP-9 inhibitor and MMP-8 resulted in more complete re-epithelialization than seen with either of the two agents alone or with vehicle (Fig. 2*A*) and in substantial reduction in apoptotic cells relative to the other three groups (Fig. 2*B*). In situ zymography with DQ-gelatin showed inhibition of MMP-9 in the groups treated with ND-336 and with the combination of ND-336 and MMP-8 (Fig. 2*C*, *Left*). In situ zymography with DQ-collagen indicated the presence of MMP-8 in the vehicle- and ND-336–treated groups, but significantly increased MMP-8 activity was found in the groups treated with MMP-8 and with the combination of ND-336 and MMP-8 (Fig. 2*D*, *Left*).

MMP-9 Inhibition and Exogenously Added MMP-8 Decrease Inflammation and Enhance Angiogenesis. Inflammation is necessary for normal wound healing. However, increased or prolonged inflammation has been shown to delay wound healing in nondiabetic mice (22). IL-6, a proinflammatory cytokine (23), plays a crucial role in the inflammatory response in wound repair (24). IL-6–deficient mice display impaired wound healing that is reversed with the administration of IL-6 (25). Delayed wound healing in IL-6–knockout mice was accompanied by attenuated leukocyte infiltration, re-epithelialization, angiogenesis, and collagen accumulation (26). TGF-β1 is a cytokine that elicits recruitment of inflammatory cells during wound healing (27). TGF-β1 is up-regulated during wound healing, suggesting that it regulates wound repair (28). Immunodeficient TGF-β1–knockout mice show delayed wound healing, with accompanying delays in the inflammatory, proliferation, and maturation phases of wound healing (27). TGF-β induces pro-MMP-9 in human skin (29), and TGF-β1 stimulates the production of MMP-9 in human corneal epithelial cells (30) and in human keratinocytes (31). Enhanced TGF-β1 signaling accelerates re-epithelialization (32). These studies suggest that IL-6 and TGF-β1 play important roles in wound healing. Therefore we measured the concentrations of IL-6 and TGF-β1 by ELISA in wounds of *db*/*db* mice.

In vehicle-treated *db*/*db* mice, IL-6 was elevated throughout the course of the study (Fig. 3*A*). Treatment with either ND-336 or MMP-8 significantly reduced IL-6, and the combination of ND-336 and MMP-8 decreased IL-6 more than either agent alone (Fig. 3 *A* and *B*). Likewise, treatment with ND-336, MMP-8, or combined ND-336 and MMP-8 significantly reduced the levels of TGF-β1 (Fig. 3 *C* and *D*).

Angiogenesis is essential for wound healing (33), facilitating the removal of debris and the development of granulation tissue that help wound closure. CD31 is found on the surface of endothelial cells and is widely used as a marker for angiogenesis. Using anti-CD31 antibodies, we found increased angiogenesis in the groups treated with ND-336, MMP-8, or combined ND-336 plus MMP-8 (Fig. S7). We used a second angiogenesis marker, VEGF, which enhances vascular permeability, promoting the formation of new blood vessels (34). The levels of VEGF were determined by ELISA as a function of time after wound infliction. Treatment with ND-336, MMP-8, or combined ND-336 and MMP-8 increased VEGF concentrations in the wounds compared with vehicle treatment (Fig. 3 *E* and *F*). Our results are in agreement with the increased VEGF levels seen in human wound fluid (35) and in epidermal keratinocytes (36).

In summary, we have shown that the novel MMP-9 inhibitor ND-336 accelerates diabetic wound healing by decreasing inflammation and by enhancing angiogenesis and re-epithelialization of the wound, thus reversing the pathological condition. Topical administration of active recombinant MMP-8 accelerated diabetic wound healing, resulting in complete re-epithelialization, diminished inflammation, and enhanced angiogenesis. The combination of a selective MMP-9 inhibitor with added MMP-8 was the best strategy to accelerate diabetic wound healing and holds promise for the treatment of diabetic wounds.

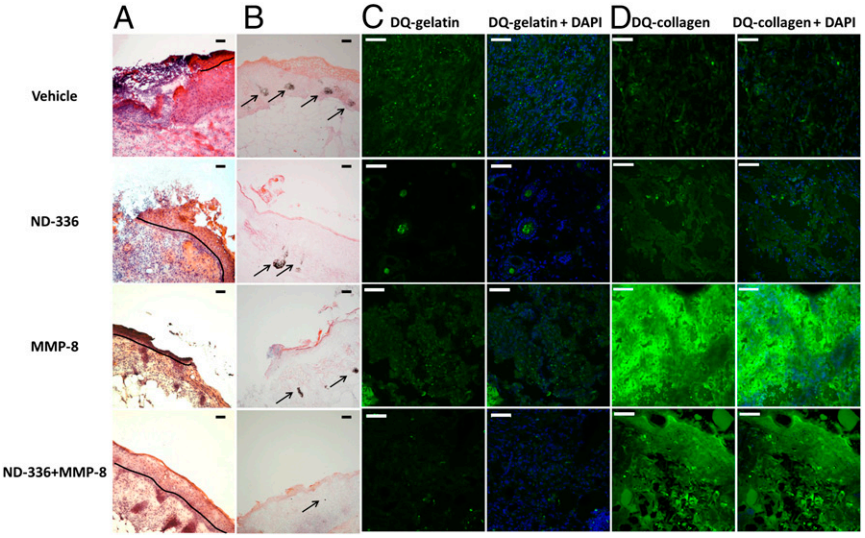

Fig. 2. Effect of MMP-9 inhibition and exogenous MMP-8 on diabetic wound healing. Mice received a single 8-mm excisional wound on the dorsal thorax. Wounds were treated with vehicle, ND-336 (0.05 mg per wound per day), MMP-8 (1 μg per wound per day), or ND-336 (0.05 mg per wound per day) plus MMP-8 (1 μg per wound per day). H&E staining, TUNEL, and in situ zymography with DQ-gelatin and DQ-collagen were performed on day 14 (n = 3 mice per group). (*A*) H&E staining for representative wounds on day 14. Re-epithelialization is indicated by the black line. Pictures were taken with a 10× lens. (Scale bars, 50 μm.) (*B*) TUNEL images of representative wounds on day 14. Arrows point to representative TUNEL$^+$ (apoptotic) cells. Pictures were taken with a 10× lens. (Scale bars, 50 μm.) (*C*) In situ zymography with gelatinase fluorogenic substrate DQ-gelatin (*Left*, green) and merged with nuclear DNA staining by DAPI (*Right*, blue). Pictures were taken with a 40× lens. (Scale bars, 50 μm.) (*D*) In situ zymography with collagenase fluorogenic substrate DQ-collagen (*Left*, green) and merged with nuclear DNA staining by DAPI (*Right*, blue). Pictures were taken with a 40× lens. (Scale bars, 50 μm.)

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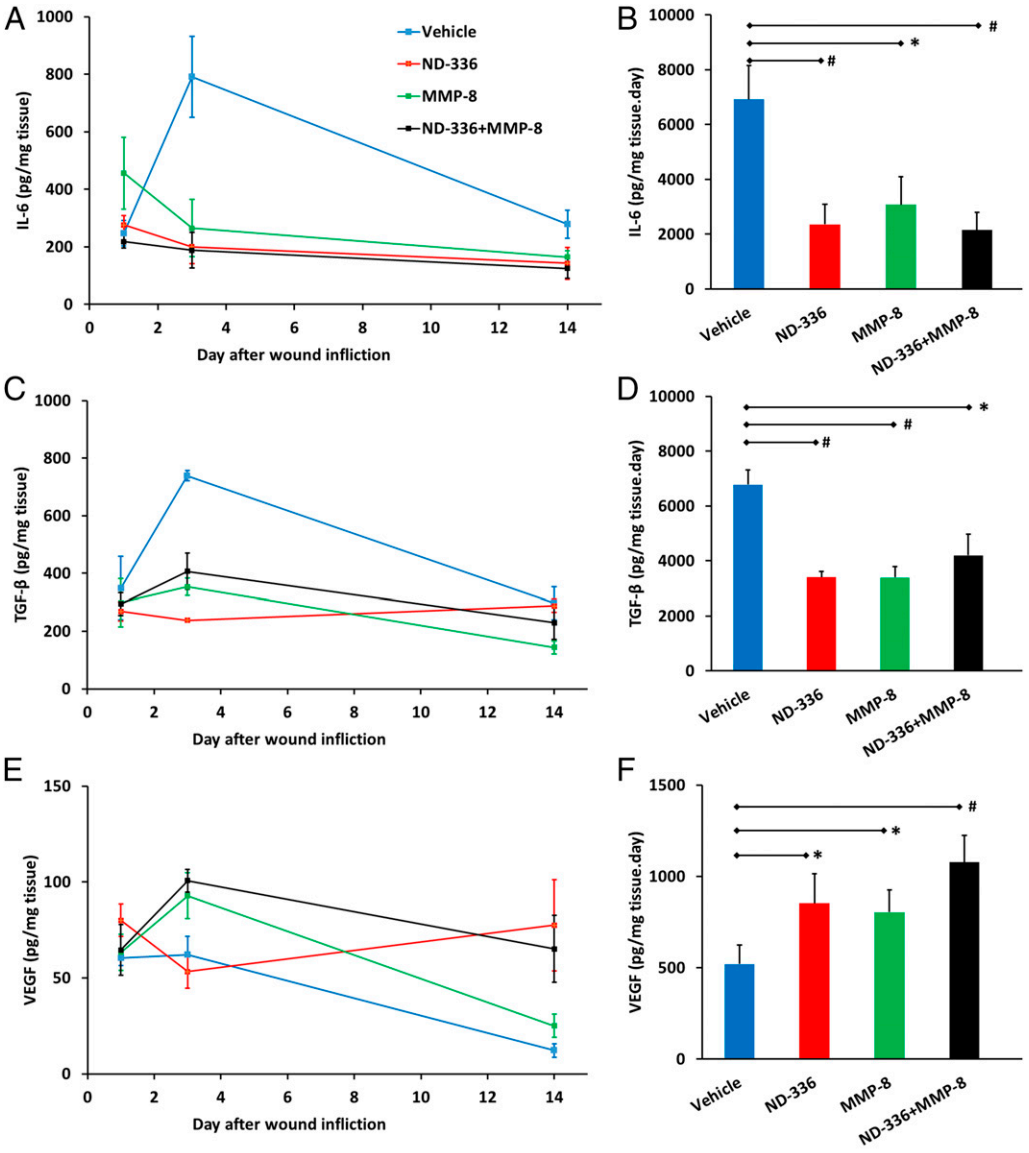

Fig. 3. MMP-9 inhibition and/or exogenous MMP-8 result in decreased inflammation and increased angiogenesis. Data represent the mean ± SD ($n = 3$ mice per group per time point; total, 36 mice). $^{*}P < 0.05$ and $^{\#}P < 0.01$ indicate statistically significant differences between the indicated groups. Statistical significance was evaluated by the Student's *t* test using a two-tail distribution and unequal variance. (*A*) Concentrations of IL-6 as a function of time after wound infliction. (*B*) The AUC for IL-6 showed that IL-6 levels were reduced significantly upon treatment with ND-336, MMP-8, or combined ND-336 and MMP-8. (*C*) Concentrations of TGF-β1 as a function of time after wound infliction. (*D*) The AUC for TGF-β1 showed that TGF-β1 levels were reduced significantly upon treatment with ND-336, MMP-8, or combined ND-336 and MMP-8. (*E*) Concentrations of VEGF as a function of time after wound infliction. (*F*) AUC for VEGF showed that VEGF levels were increased significantly upon treatment with ND-336, MMP-8, or combined ND-336 and MMP-8.

Materials and Methods

Synthesis of ND-336. Detailed procedures for the synthesis of ND-336 are given in *SI Materials and Methods*.

Synthesis of ND-322. ND-322 was synthesized as previously reported (10).

Enzyme Inhibition Studies. Human recombinant active MMP-2 and MMP-7 and the catalytic domains of MMP-3 and MMP-14/MT1-MMP were purchased from EMD Chemicals, Inc.; human recombinant catalytic domains of MMP-1, MMP-8, and MMP-9 were purchased from Enzo Life Sciences, Inc.; human recombinant active ADAM9 and ADAM10 were purchased from R&D Systems. Fluorogenic substrates (7-methoxycoumarin-4-yl)acetyl (Mac)-Pro-Leu-Gly-Leu-[Nβ-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl](Dap)(Dnp)-Ala-Arg-NH_2 (for MMP-2, MMP-7, MMP-9, and MMP-14) and Mac-Arg-Pro-Lys-Pro-Val-Glu-norvaline (Nva)-Trp-Arg-Lys(Dnp)-NH_2 (for MMP-3) were purchased from Peptides International; Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (for MMP-1, MMP-8, and ADAM10) and Mac-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2 (for ADAM9) were purchased from R&D Systems. The K_m values for MMP-2, MMP-9, and MMP-14 were the same as previously reported by Gooyit et al. (37). Inhibitor stock solutions (10 mM) were prepared freshly in DMSO before enzyme inhibition assays. We followed the methodology for enzyme inhibition studies as reported previously by Page-McCaw, et al. (38). Enzyme inhibition studies were carried out using a Cary Eclipse fluorescence spectrophotometer (Varian). Compound **1** was stable in the buffers used in the kinetic assays.

Animals. Female diabetic *db/db* mice (BKS.Cg-*Dock7*$^{m\ +/+}$ *Lepr*^{db}/J, 8 wk old) were purchased from the Jackson Laboratory and were fed 5001 Laboratory Rodent Diet (LabDiet) and water ad libitum. Mice were housed in polycarbonate shoebox cages with hardwood bedding at 72 ± 2 °F and 12-h/12-h light/dark cycles. All procedures involving vertebrate animals were approved by the Institutional Animal Care and Use Committee at the University of Notre Dame.

Excisional Diabetic Wound Model. The dorsal area of the mice was shaved, and a single excisional wound 8 mm in diameter was made on the dorsal thorax with a biopsy punch (Miltex) while the animals were under isofluorane anesthesia. Wounds were covered with Tegaderm dressing (3M Company). Topical treatment was started the next day.

Wound Measurements. Mice were anesthetized with isofluorane, and wounds were photographed with an Olympus SP-800 UZ camera mounted on a tripod at a fixed distance; a ruler was included in the digital photo. Wound areas were calculated using NIH ImageJ software (version 1.48) and expressed as percent change in wound area relative to day 0.

ND-336 Diabetic Wound-Healing Study. For the ND-336 diabetic wound-healing study, *db/db* mice were divided into three groups ($n = 8$ mice per group, total 24 mice): vehicle-treated (50 μL of 20% DMSO, 80% propylene glycol per wound per day), ND-322–treated (50 μL of a 2-mg/mL solution of ND-322 in 20% DMSO/80% propylene glycol, equivalent to 0.1 mg per wound per day),

and ND-336–treated (50 μL of a 2 mg/mL solution of ND-336 in 20% DMSO/80% propylene glycol, equivalent to 0.1 mg per wound per day). Wound measurements were taken on days 0, 7, 10, and 14. Animals were killed on day 14, and wounds were embedded in optimal cutting temperature (OCT) compound, cryosectioned, and analyzed by H&E staining, TUNEL, and in situ zymography.

Exogenous MMP-8 Study. Female diabetic *db/db* mice (BKS.Cg-*Dock7m* $^{+/+}$ *Leprdb*/J, 8 wk old, 38 ± 3 g, $n = 40$) were used for the exogenous MMP-8 study. Wounds were inflicted as described, and on the next day the wounds were treated topically with MMP-8 (50 μL of 20 μg/mL MMP-8 in reaction buffer) or vehicle (50 μL reaction buffer) once each day for 14 d. The reaction buffer consisted of 50 mM Tris (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% (wt/vol) Brij-35. Digital photographs of the wounds were taken on days 0, 7, 10, and 14 while animals were under isoflurane anesthesia. On days 7 and 14, 20 mice (n = 10 per group) were killed. The wounds were excised, embedded in OCT compound, and cryosectioned for histological evaluation and in situ zymography.

Combined ND-336 and MMP-8 Study. In the combined ND-336 and MMP-8 study, female *db/db* mice ($n = 51$) were divided into four groups: vehicle -treated [50 μL of 10% DMSO/10% propylene glycol/80% saline per wound per day dosed in the morning and 50 μL of reaction buffer 50 mM Tris (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% (wt/vol) Brij-35) per wound per day dosed in the afternoon], ND-336–treated [50 μL of 1 mg/mL ND-336 (equivalent to 0.05 mg per wound per day) in 10% DMSO/10% propylene glycol/80% saline dosed in the morning and 50 μL of reaction buffer dosed in the afternoon], MMP-8–treated [50 μL of 10% DMSO/10% propylene glycol/80% saline per wound per day dosed in the morning and 50 μL of 20 μg/mL of MMP-8 in reaction buffer (equivalent to 1 μg per wound per day) dosed in the afternoon], and combined ND-336– and MMP-8–treated (0.05 mg of ND-336 in 50 μL of 10% DMSO/10% propylene glycol/80% saline per wound per day dosed in the morning and 1 μg of MMP-8 in 50 μL of reaction buffer per wound per day dosed in the afternoon). Mice were killed on days 7, 10, and 14, and the excised wounds were embedded in OCT compound and cryosectioned for histological evaluation and in situ zymography.

Measurement of IL-6, TGF-β1, and VEGF by ELISA. Wound tissues ($n = 3$ mice per group) were harvested and were frozen immediately in liquid nitrogen on days 1, 3, and 14. The extracted tissues were homogenized in cold lysis buffer containing EDTA-free protease inhibitor mixture (Pierce). The lysates were analyzed for protein concentration by the Bradford protein assay (Bio-Rad). The levels of IL-6, TGF-β1, and VEGF in the lysates were determined by ELISA, following the manufacturer's protocol (Abcam). The cytokine levels for each mouse sample were expressed in picograms per milligram of tissue. The area under the curve (AUC) was calculated by the linear trapezoid rule using GraphPad Prism 5 for Windows Version 5.01 (GraphPad Software, Inc.). AUC is reported as mean ± SD.

Statistical Analyses. Data were analyzed for statistical significance using the two-tailed Mann–Whitney *u* test (GraphPad Prism 5). Inflammation and angiogenesis markers were analyzed for statistical significance by the Student *t*-test (Excel) using a two-tail distribution and unequal variance because the Mann–Whitney *u* test will always give a *P* value greater than 0.05 regardless of the groups differences when the total sample size is seven or less.

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