Inhibitors of Dipeptidyl Peptidase IV-Like Activity Mediate Antifibrotic Effects in Normal and Keloid-Derived Skin Fibroblasts

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Suppression of collagen and matrix synthesis and inhibition of the fibrogenic cytokine transforming growth factor-β_1 (TGF-β_1) is a major therapeutic goal in the treatment of fibrosis and keloids. Inhibitors of dipeptidyl peptidase IV (DP IV)-like activity affect cell growth and cytokine production and are currently under investigation for the treatment of metabolic, autoimmune and inflammatory diseases. We show here that the inhibitors of DP IV-like activity, Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-pyrrolidide, suppress proliferation in human skin fibroblasts and keloid-derived skin fibroblasts *in vitro*. They significantly decrease TGF-β_1 expression and secretion of procollagen type I C-terminal peptide in supernatants of both cell types. Furthermore, they abrogate the TGF-β_1-induced stimulation of collagen synthesis, matrix deposition, and TGF-β_1 and fibronectin expression. Both inhibitors lead to dephosphorylation of mitogen-activated protein kinases pp38 and pERK1/2, which are activated upon TGF-β1 stimulation and have been implicated in fibrogenesis. In a mouse model of dermal fibrosis, induced by repetitive intracutaneous injections of TGF-β_1, the profibrotic effect of TGF-β_1 detected by dermal thickening, collagen I, and α-smooth muscle actin expression, is significantly suppressed in the presence of inhibitors. Inhibition of DP IV-like enzymatic activity may therefore represent a promising therapeutic approach for the treatment of fibrotic skin disorders and keloids.

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INTRODUCTION

Fibrosis and sclerosis of the skin and subcutaneous tissue can be seen in a variety of disorders such as keloids, hypertrophic scars, morphea, systemic sclerosis, eosinophilic fasciitis, sclerodermic graft-versus-host disease, and other pathological conditions influenced by toxic or pharmacological factors, for example, toxic-oil-syndrome or eosinophilia myalgia. The major pathogenetic feature of fibrosis is represented by excessive accumulation of collagen and extracellular matrix (ECM), leading to progressive organ dysfunction in liver, lung, kidney, or skin (Chapman, 2004; Al Attar *et al.*, 2006; Gressner and Weiskirchen, 2006; Liu, 2006). Transforming growth factor-β_1 (TGF-β_1) has been identified as a central player in the induction and aggravation of fibrosis (Blobe *et al.*, 2000; Leask and Abraham, 2004).

In keloid-derived skin fibroblasts (KFs), TGF-β_1 expression and the response to TGF-β_1-induced stimulation of proliferation, collagen, and fibronectin production are increased as compared with normal fibroblasts (Babu *et al.*, 1992; Bettinger *et al.*, 1996; Fujiwara *et al.*, 2005). These data together with the observation that a TGF-β_1-neutralizing antibody-reduced scarring (Shah *et al.*, 1995) suggest a crucial role of TGF-β_1 in the development of keloids and scarring. Furthermore, alterations in the expression and signalling of this cytokine have been implicated in scleroderma pathogenesis (Varga and Abraham, 2007). Therefore, therapeutic strategies antagonizing the strong fibrogenic effect of TGF-β_1 are regarded as a promising approach to prevent the development and progression of keloids, scleroderma, and other fibrotic disorders (Gressner and Weiskirchen, 2006; Leask, 2006).

The dipeptidyl peptidase IV (EC 3.4.14.5, dipeptidyl peptidase IV (DP IV), CD26) is a homodimeric type II transmembranic glycoprotein (Fleischer, 1994) that belongs to the group of postproline dipeptidyl aminopeptidases

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Abbreviations: DP IV, dipeptidyl peptidase IV; ECM, extracellular matrix; FAP-α, fibroblast activation protein-α; KF, keloid-derived skin fibroblast; LZNP, Lys[Z(NO_2)]-pyrrolidide; LZNT, Lys[Z(NO_2)]-thiazolidide; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase NF, normal skin fibroblast; PBS, phosphate-buffered saline pERK, phospho-extracellular signal-related kinase; PICP, procollagen type I C-terminal peptide; TBST, Tris-buffered Saline Tween 20; TGF-β_1, transforming growth factor-β_1; TIMP, tissue inhibitor of metalloproteinase

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consisting of the gene family of DP IV; fibroblast activation protein-α (FAP-α); dipeptidyl peptidases DP8 and DP9; and DP II (Gorrell, 2005). It is a serine exopeptidase catalyzing the release of N-terminal dipeptides from oligo- and polypeptides preferentially with proline in the penultimate position, which results in playing a key role in the catabolism of a number of neuropeptides, immunopeptides and peptide hormones (De Meester *et al.*, 1999; Lambeir *et al.*, 2003). Furthermore, DP IV functions as a binding protein for fibronectin, collagen and adenosin deaminase and, in association with seprase (FAP-α), plays a role in tissue invasion and matrix degradation (Ghersi *et al.*, 2002). Interestingly, it was found that the pharmacological inhibition of DP IV-like activity affects growth, cytokine production, and typical functions of peripheral T cells (Reinhold *et al.*, 2002), keratinocytes, and sebocytes *in vitro* (Reinhold *et al.*, 1998; Thielitz *et al.*, 2007).

In vivo, inhibitors of DP IV-like activity have potent immunosuppressive and anti-inflammatory effects in many pathological conditions such as murine experimental autoimmune encephalomyelitis (Steinbrecher *et al.*, 2001), collagen- and alkyldiamone-induced arthritis (Tanaka *et al.*, 1997), or rat cardiac and lung transplantation (Korom *et al.*, 1997; Jung *et al.*, 2006). Currently, the clinical efficacy of DP IV inhibitors is under investigation for the treatment of type 2 diabetes, as well as multiple sclerosis, rheumatoid arthritis, colitis ulcerosa, psoriasis, and acne.

A major concern for the systemic use of inhibitors of DP IV-like activity is the possible induction of fibrosis via the temporary induction of TGF-β_1 in T cells (Reinhold *et al.*, 1997). However, despite the fact that TGF-β_1 plasma levels were increased and discussed to be responsible for various immunosuppressive effects, no fibrotic conditions have been observed in treated animals (Steinbrecher *et al.*, 2001). To date, only few investigations are available on the effects of DP IV inhibitors on fibroblast activity. Most importantly, it is not known whether inhibition of DP IV-like activity induces pathological changes in fibroblast function that could result in the generation of fibrosis. A previous investigation suggesting a suppressive effect of DP IV inhibitors on platelet-derived growth factor-stimulated fibroblast activity (Williams *et al.*, 2003) prompted us to deeper investigate the role of inhibitors of DP IV-like activity in skin fibroblast biology. This is also of interest because these substances are discussed as promising tools for the treatment of inflammatory or hyperproliferative skin diseases.

Here we show that the inhibitors of DP IV-like activity, Lys[Z(NO_2)]-thiazolidide (LZNT) and Lys[Z(NO_2)]-pyrrolidide (LZNP), suppress proliferation, TGF-β_1 and fibronectin expression, procollagen type I C-terminal peptide (PICP) secretion, as well as collagen and matrix deposition of normal skin fibroblasts (NFs) and KFs *in vitro*. Furthermore, they abrogate TGF-β_1-induced profibrotic effects *in vitro* and, as shown in a mouse model of cutaneous fibrosis, *in vivo*.

Our data provide early evidence that DP IV-like activity is involved in the regulation of major fibroblast functions, and may represent a promising pharmacological target in the treatment of keloids and fibrotic or sclerotic diseases.

RESULTS

Expression of DP IV and related enzymes on NFs and KFs skin fibroblasts

As it is known that DP IV is upregulated upon activation in T cells and keratinocytes (Novelli *et al.*, 1996; De Meester *et al.*, 1999), we were interested to see whether DP IV expression was altered in KFs compared with NFs. We investigated DP IV expression of NFs and KFs of six different donors grown by explant cultures. Flow cytometric analysis revealed that 73±16% of NF express DP IV, a proportion significantly higher ($P<0.01$) than that of KF (46±25%) (Figure 1b). Accordingly, the DP IV-like enzymatic activity (Figure 1a) detected by Gly–Pro–pNA-hydrolyzing activity was also higher in NF (83±24 pkat/10^6 cells) as compared with KF (48±26 pkat/10^6 cells). Gly–Pro–pNA is also hydrolyzed by DP8 and DP9, which are localized in the cytoplasm, and, with a 100-fold weaker catalytic efficiency (Aertgeerts *et al.*, 2005), by FAP-α. We found no difference in the qualitative mRNA expression of DP IV-like proteins FAP-α, DP 8, DP 9 between NF and KF of five different donors (Figure 1c). In cell suspensions of permeabilized cells as well as in cell homogenates, we found no significant difference of DP IV-like activity and its suppression by LZNP and LZNT as compared with native cells. Thus, we conclude that both the substrate and the inhibitors enter the cells and that the DP IV-like enzyme activity we measure results from DP IV on the surface, dipeptidyl peptidase 8 and/or 9 in the cytoplasm, and, to a negligible extent, from FAP-α. Surface expression of FAP-α was equal in both cell types (49±10 *vs* 51±6%, respectively; data not shown).

Inhibitors of DP IV-like activity suppress enzymatic activity and proliferation of NF and KF *in vitro*

We found that the inhibitors LZNT and LZNP, used in concentrations of 0.1–10 μM, significantly ($P<0.05$) suppress enzymatic activity of NF (Figure 2a) and KF (Figure 2b) in a dose-dependent manner. Inhibitors of DP IV-like activity are

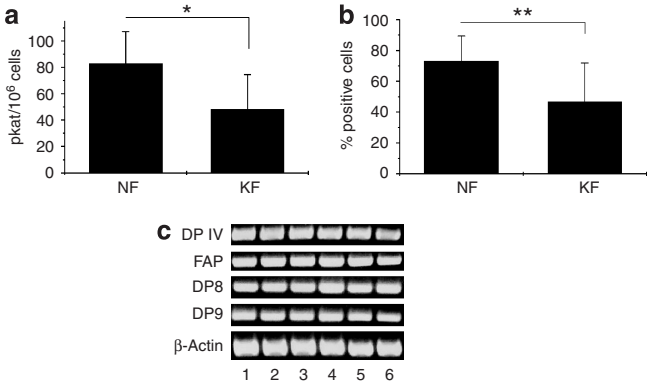


Figure 1. Expression of DP IV/CD26 and related enzymes on NF and KFs. (**a**) The DP IV-like activity was measured by Gly–Pro–4-nitroanilide hydrolysis. (**b**) DP IV surface expression was detected by flow cytometry. The data are presented as mean±SD ($n=6$ in each group; *$P<0.05$, **$P<0.01$, Student's *t*-test). (**c**) mRNA expression of DP IV and DP IV-like enzymes. Enzymatic amplification was performed on cDNA derived from 70–80% confluent cell cultures (1 = NF; 2–6 = KF of five different donors).

known to influence proliferation in various cell types. Therefore, we were interested to test whether the proliferation of fibroblasts was also suppressed in the presence of our inhibitors.

The DNA synthesis of normal and KFs was dose dependently suppressed after 24 hours of incubation (Figure 2c and d), in the presence of different inhibitor concentrations (3.125–50 μM), as compared with control cultures without inhibitor. In both cell types, effects on DNA synthesis were only moderate with a maximum of 30–40% inhibition.

A possible cytotoxic effect of the inhibitors at the concentrations used could be excluded using the lactate dehydrogenase cytotoxicity detection assay, and Trypan blue staining demonstrated that more than 95% of cells are viable in all culture systems 6 hours after incubation (data not shown).

Inhibitors of DP IV-like activity decrease TGF-β_1 production in NFs and KFs

An important property of TGF- β_1 in the induction of fibrosis is its capability to activate its own mRNA expression and thereby increase its own secretion (Kim *et al.*, 1990). We show here that basal production of latent TGF-β_1 is significantly suppressed in the presence of LZNP and LZNT after 72 hours of incubation in both NF ($P<0.05$) and KF ($P<0.001$) (Figure 3). The decrease was clearly time dependent, with a minor effect already after 24 hours, a significant, but modest effect after 48 hours (data not shown), and a maximum effect after 72 hours. To mimic pathological conditions in which fibrosis is induced by increased TGF-β_1 expression, we stimulated our cultures by the addition of 10 ng/ml of TGF-β_1. As shown in Figure 3, 72 hours after stimulation the TGF-β_1 expression in supernatants was increased 4- to 5-fold. Control experiments were performed to exclude that the TGF-β_1 measured was the originally added ''active'' cytokine. Active TGF-β_1 could be detected only in the first five minutes after it was added to the cultures,

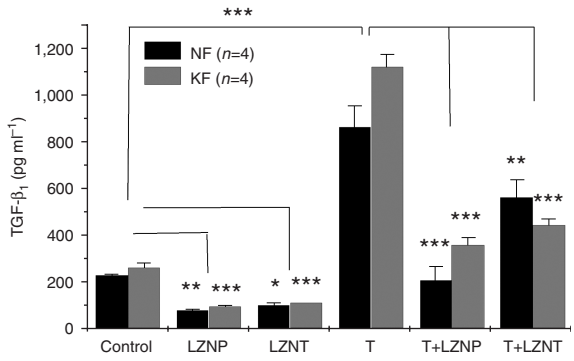


Figure 3. DP IV inhibitors induce decreased production of latent TGF-β_1 in NFs and KFs. NFs and KFs were incubated with the inhibitors LZNT and LZNP (50 μM), or with TGF-β_1 (T) (10 ng/ml), or TGF-β_1 and one inhibitor. After 72 hours, supernatants were harvested and the production of latent TGF-β_1 was measured. TGF-β_1 alone used as autostimulation factor of TGF-β_1 production served as a positive control. The data represent the mean ± SEM of four independent experiments. Statistics were calculated by one-way analysis of variance (*$P<0.05$, **$P<0.01$, ***$P<0.001$, Tukey's multiple comparison test).

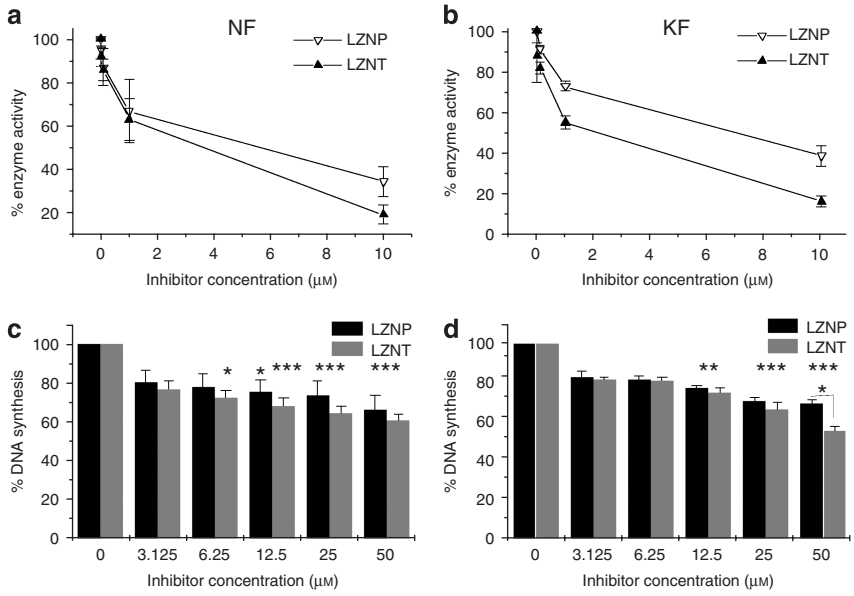


Figure 2. Suppression of enzymatic activity and proliferation of NFs and KFs in the presence of inhibitors. The inhibitors of DP IV-like activity, LZNP and LZNT, dose dependently reduce enzymatic activity (Gly–Pro–4-nitroanilide hydrolysis) in NF (**a**) and KF (**b**) (both $P<0.05$). The data are presented as mean ± SEM of four independent experiments. (**c** and **d**) The DNA synthesis measured by [^{3}H]thymidine incorporation was significantly suppressed compared with control cultures in NFs (**c**) and KFs (**d**) at concentrations of 12.5 μM or higher. The DNA synthesis values are expressed as percentage of [^{3}H]thymidine incorporation in relation to control cultures without inhibitor (100% = 5,691 ± 2,300 c.p.m. (NF) or 100% = 3,167 ± 674 (KF)), and represent the mean ± SEM of six independent experiments. Statistics were calculated by linear regression analysis (**a** and **b**) or by one-way analysis of variance (*$P<0.05$, **$P<0.01$ *vs* control, Tukey's multiple comparison test).

thus confirming that the cytokine measured after 72 hours represents the latent TGF-β_1 produced by the cultured cells (data not shown). The inhibitors of DP IV-like activity significantly abrogate the TGF-β_1-induced stimulation of TGF-β_1 autoproduction both in NFs ($P<0.01$) and KFs ($P<0.001$) (Figure 3).

We have next investigated whether the modulatory activity of inhibitors of DP IV-like activity on TGF-β_1 expression is regulated at the transcriptional level. NFs were incubated with DP IV inhibitors LZNT or LZNT for 4, 24, and 48 hours. The relative mRNA levels of TGF-β_1 were subsequently determined by quantitative real-time PCR. Neither LZNT nor LZNP caused a significant reduction in the relative levels of the TGF-β_1 mRNA at 4 or 24 hours, thus suggesting that inhibitors of DP IV-like activity do not directly regulate TGF-β_1 transcription. However, we found a significant suppression of TGF-β_1 mRNA after 48 hours (data not shown).

Inhibition of DP IV-like activity leads to suppression of collagen and matrix deposition in TGF-β_1-stimulated fibroblasts

Increased collagen and matrix deposition are key features of fibrosis and keloids. [^{3}H]Proline incorporation assays were performed to characterize the ECM production by NFs and KFs. No significant differences were found between basal production of NFs and KFs, and between control cultures and inhibitor-treated cultures (Figure 4a and b). After stimulation with 10 ng/ml TGF-β_1, in both cell types a strong increase of collagen (NF $\times$ 3; KF $\times$ 4) and matrix deposition (NF $\times$ 1.5; KF $\times$ 3) was found, which was more pronounced in KFs. When LZNP and LZNT were added together with TGF-β_1, the TGF-β_1-induced

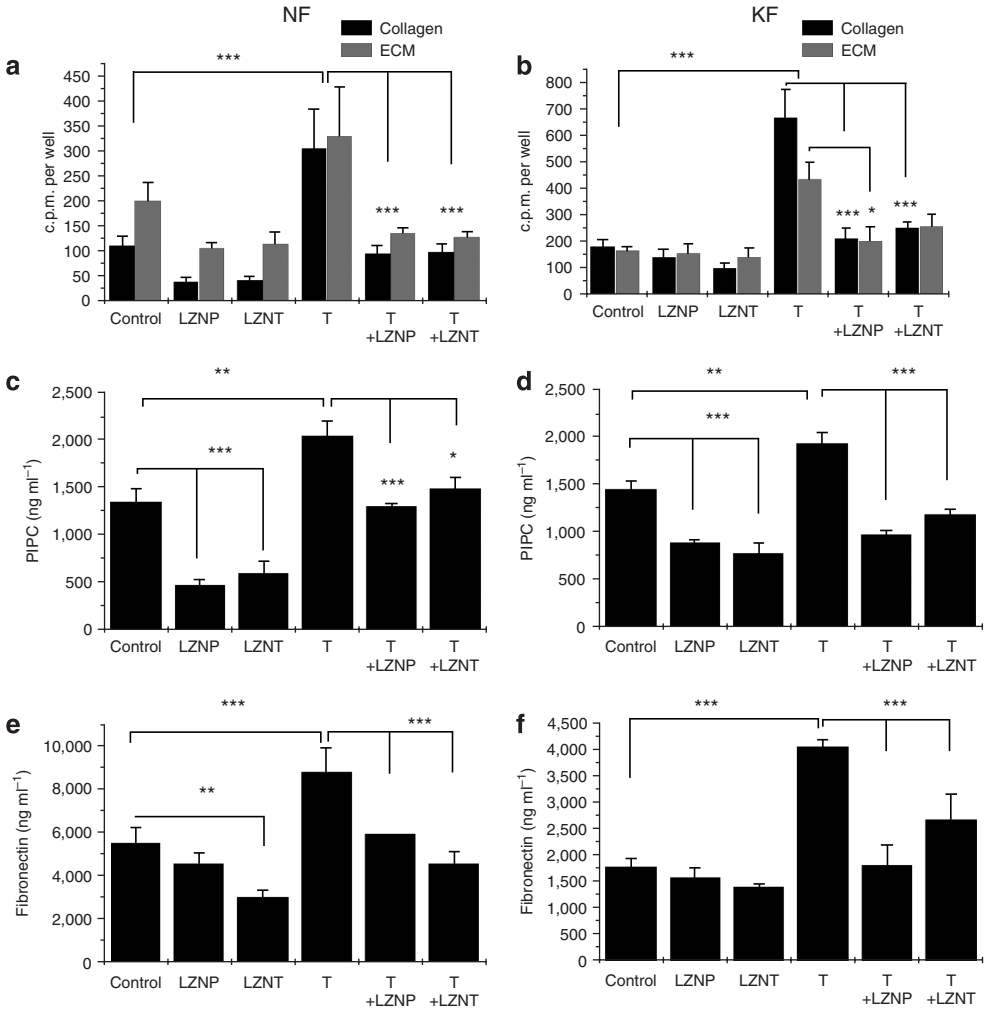


Figure 4. Suppression of collagen and matrix deposition, PICP synthesis, and fibronectin expression in NFs and KFs. (**a** and **b**) Regulation of collagen and matrix deposition in NFs (**a**) and KFs (**b**) in the presence of inhibitors LZNT or LZNP (50 μM), and after stimulation with TGF-β_1 (T) at 10 ng/ml in the presence or absence of inhibitors ($t=72$ hours). The data represent the mean $\pm$ SEM of six independent experiments. (**c** and **d**) Inhibitor-mediated suppression of PICP secretion induced by TGF-β_1 in NF (**c**) or KF (**d**). (**e** and **f**) Inhibitor-mediated suppression of fibronectin expression induced by TGF-β_1 in NF (**e**) or KF (**f**). NF or KF were left untreated or incubated for 72 hours with 10 ng/ml TGF-β_1 (T), inhibitors LZNP or LZNT at 50 μM, or both substances. Statistics were calculated by one-way analysis of variance (*$P<0.05$, **$P<0.01$, ***$P<0.001$, Tukey's multiple comparison test).

stimulation was completely and significantly abrogated in both cell types.

Inhibition of DP IV-like activity leads to suppression of collagen synthesis

To further substantiate the effects of inhibitors of DP IV-like activity on collagen synthesis and/or secretion, we determined the amount of PICP in the culture media of NFs or KFs incubated with TGF-β_1 and DP IV inhibitors LZNT or LZNP alone, or a combination of TGF-β_1 and one of the inhibitors. TGF-β_1 significantly ($P<0.01$) stimulated PICP secretion as compared with control cultures in NFs ($1,350\pm311$ *vs* $2,042\pm340$ ng/ml) and KFs ($1,459\pm166$ *vs* $1,933\pm270$ ng/ml).

We found that the PICP secretion by NFs and KFs was significantly lowered in the presence of inhibitors alone compared with control cultures without inhibitor or cultures stimulated with TGF-β_1 (Figure 4c and d). Quantitative real-time PCR experiments showed no significant suppression of mRNA production of collagens I ($\alpha1$ and $\alpha2$) and III ($\alpha1$) after 12 hours incubation in the presence of inhibitors (data not shown).

Inhibition of DP IV-like activity suppresses the TGF-β_1-induced stimulation of fibronectin expression

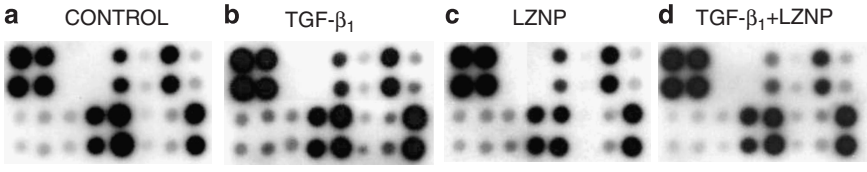
To further elucidate inhibitor effects on ECM, we investigated fibronectin expression in culture supernatants obtained 72 hours after inhibitor or TGF-β_1 were added to the cultures. No significant differences were found between basal fibronectin expression in NF and KF supernatants and between control cultures and inhibitor-treated cultures, except for LZNT-treated cultures in NF (Figure 4e and f). After stimulation with 10 ng/ml TGF-β_1, in both cell types a significant increase of fibronectin (NF $\times$ 1.6; KF $\times$ 2.3) was found, which was more pronounced in KFs. When LZNP and LZNT were added together with TGF-β_1, the TGF-β_1 induced stimulation was significantly suppressed in both cell types.

DP IV inhibitors abrogate the TGF-β_1-induced alteration of MMP expression

TGF-β_1 is known to regulate matrix- and collagen-degrading proteases (Ravanti *et al.*, 1999; Saed *et al.*, 2000). Using a commercially available matrix metalloproteinase (MMP) antibody array, we next investigated whether the expression of MMPs, or their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), is influenced by inhibitors of DP IV-like activity, and thereby contributes to the observed inhibitor effects on collagen and matrix deposition. Figure 5 shows the results of the MMP antibody array from NFs left untreated or incubated with TGF-β_1, the inhibitor LZNP, or a combination of both. Similar experiments were performed with KFs and the other inhibitor, LZNT. A relative semi-quantitative analysis of dot size and intensity (summarized in Table 1) revealed that TGF-β_1 upregulates several metalloproteases (MMP-2, MMP-8, MMP-10, MMP-13) in KFs and NFs. In the presence of inhibitors of DP IV-like activity, the stimulatory effect of TGF-β_1 on MMP-2 and MMP-13 expression was diminished, whereas inhibitors alone slightly suppressed MMP-1 and MMP-2 expression. We found no relevant changes in the expression of TIMPs 1–4.

Inhibition of DP IV-like activity leads to dephosphorylation of p38-MAPK and pERK1/2

To further clarify the underlying mechanisms of the relationship between DP IV-like activity and fibroblast functions, we next investigated whether the phosphorylation status of the mitogen-activated protein kinases (MAPKs) p38 and ERK1/2 is influenced in the presence of inhibitors. The rationale for these experiments was that inhibitors of DP IV-like activity were previously shown to affect ERK1/2 and p38 activation in lymphocytes (Kähne *et al.*, 1999), and that both kinases were implicated in the regulation of collagen and matrix metabolism (Sato *et al.*, 2002; Li *et al.*, 2006). We first performed experiments to investigate time-dependent effects (data not shown) and found that a maximum suppression of p38 and ERK1/2 phosphorylation was observed 60–120 minutes after the DP IV inhibitors LZNP and LZNT were added. At the same time points, maximum stimulatory effects on p38 and ERK1/2 phosphorylation were observed after addition of TGF-β_1. Figure 6 shows that inhibition of DP IV-like activity downregulates phospho-p38 (pp38) and phospho-extracellular signal-related kinase 1/2 (pERK1/2) compared with unstimulated control cells, and abrogates the TGF-β_1-induced stimulation of the phosphorylation of both kinases ($t=90$ minutes). This is shown in NFs (Figure 6a) and KFs (Figure 6b). As Smad3 has been shown to be a key mediator of TGF-β_1-induced fibrotic effects, we also investigated



POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-3	TIMP-4	POS
MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-3	TIMP-4	POS

Figure 5. Results of the human MMP antibody array. NFs were cultured in DMEM supplemented with 0.1% FCS and left untreated (**a**), treated with TGF-β_1 10 ng/ml(**b**), with LZNP (50 μM) (**c**) or a combination of TGF-β_1 and LZNP (**d**). Supernatants obtained after 72 hours of incubation were analyzed by the MMP antibody array (layout listed above).

Table 1. Results of a semi-quantitative comparative analysis of MMP array membranes

	MMP-1	MMP-2	MMP-3	MMP-8	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-3	TIMP-4
NF											
TGF-β1	↔	↑	↔	↑↑	↔	↑	↑	↔	↔	↔	↔
LZNP	↓	↓↓	↔	↔	↔	↔	↔	↔	↓	↓↓	↔
LZNT	↓	↓	↔	↔	↔	↔	↔	↔	↔	ND	↔
TGF-β1+LZNP	↓	↔	↓	↔	↓	↔	↓	↔	↔	↔	↔
TGF-β1+LZNT	↓	↔	↓	↔	↔	↔	↓	↔	↔	ND	↔
KF											
TGF-β1	↔	↑	↔	↑	↔	↑	↑	↔	↔	↑	↓
LZNP	↔	↓	↔	↔	↔	↔	↔	↔	↔	↔	↔
LZNT	↓	↓↓	↔	↓	↓	↔	↓	↓	↔	ND	↓
TGF-β1+LZNP	↔	↔	↔	↔	↔	↔	↔	↔	↔	↑	↓
TGF-β1+LZNT	↓↓	↔	↔	↔	↔	↔	↔	↔	↔	ND	↔

KF, keloid-derived skin fibroblast; LZNP, Lys[Z(NO_2)]-pyrrolidide; LZNT, Lys[Z(NO_2)]-thiazolidide; MMP, matrix metalloproteinase; NF, normal skin fibroblast; TGF-β1, transforming growth factor-$β_1$; TIMP, tissue inhibitor of metalloproteinase.
The relative changes compared with control membranes left untreated are summarized (↔ 0.61–1.5 ×; ↑ 1.51–3.0 ×; ↑↑ >3.0 ×; ↓ 0.2–0.6 ×; ↓↓ <0.2; × =fold control; ND=not done). The framed boxes correspond to the shown membranes.

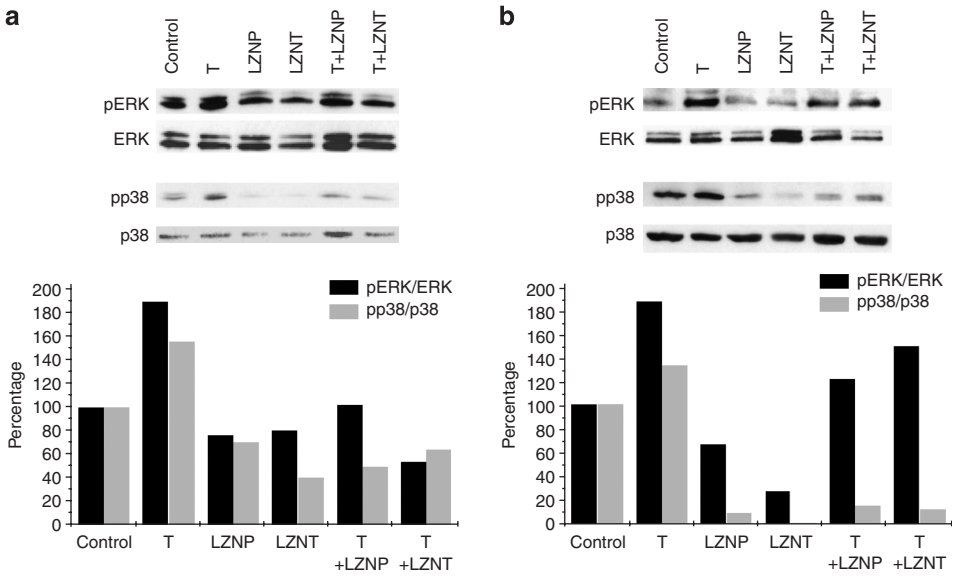


Figure 6. Effects of TGF-$β_1$ and inhibitors of DP IV-like activity on ERK and p38 phosphorylation. NF (**a**) and KF (**b**) were incubated for 90 minutes in the presence of the inhibitors LZNT and LZNP (50 μM), or with TGF-$β_1$ (T) (10 ng/ml), or TGF-$β_1$ and one inhibitor in combination, and lysed for Western blot analysis. Blots were incubated with rabbit anti-phospho-p44/42, anti-p44/42-MAPK, anti-phospho-p38 MAPK, and with anti-p38-MAPK. Experiments were repeated three times with similar results. The graph below the blots summarizes the results of the densitometric analysis.

whether pSmad3 activation was influenced in the presence of LZNP or LZNT. After 90 minutes of incubation, we found no differences between inhibitor-treated and control cultures with or without TGF-$β_1$ (data not shown). All TGF-$β_1$-treated cultures showed a strong pSmad3 activation. We conclude that our inhibitors do not act via interaction with the Smad pathway.

Antifibrotic effect of inhibitors of DP IV-like activity *in vivo*

To examine the *in vivo* relevance of our findings, we used an animal model in which cutaneous fibrosis is induced by repetitive intracutaneous injections of high doses of TGF-$β_1$ (Shinozaki *et al.*, 1997; Böhm *et al.*, 2004). In this model, newborn mice are used because of their lower collagen content in the skin compared with adult mice, thus rendering

them more sensitive for fibrogenic stimuli. Newborn mice were injected into the neck on three consecutive days with TGF-β_1 (800 ng), or LZNT or LZNP (100 μM) plus TGF-β_1, or phosphate-buffered saline (PBS). On day 4, punch biopsies were taken from the injection sites and subjected to histological analysis (Figure 7a–l). As it is likely that fibrosis contributes to increased dermal thickness, we used histometrical analysis to assess the effect of inhibitors of DP IV-like activity on TGF-β_1-induced skin fibrosis. When compared with PBS alone, injections with TGF-β_1-induced dermal thickening (Table 2) as well as increased numbers of collagen fibers as shown by Van Gieson staining and by immunohistochemistry using an anti-collagen type I antibody (Figure 7a–c *vs* d–f) and an α-smooth muscle actin antibody (Table 2). Injections with inhibitors alone did not produce significant changes as compared with mice treated with PBS/BSA (data not shown). Simultaneous injection with TGF-β_1 plus LZNP or LZNP resulted in a significant reduction in dermal thickening as compared with mice injected with TGF-β_1 alone (Figure 7d–f *vs* g–i or j–k; Table 2). These results confirm the anti-fibrogenic activity of inhibitors of DP IV-like activity *in vivo*.

DISCUSSION

The data presented here provide early evidence that inhibitors of DP IV-like activity suppress fibroblast proliferation in normal and KFs, decrease TGF-β_1 expression, and abrogate the TGF-β_1-mediated stimulatory effects on TGF-β_1 and fibronectin production, collagen synthesis, and matrix deposition.

TGF-β_1 has been identified as a key mediator in the generation of fibrosis with pleiotropic effects, among which are the increase of collagens type I, III, VI, VII, X, fibronectin, proteoglycans, fibrogenic growth factors such as connective tissue growth factor, or the inhibition of matrix degradation by regulating the synthesis of proteases and their inhibitors

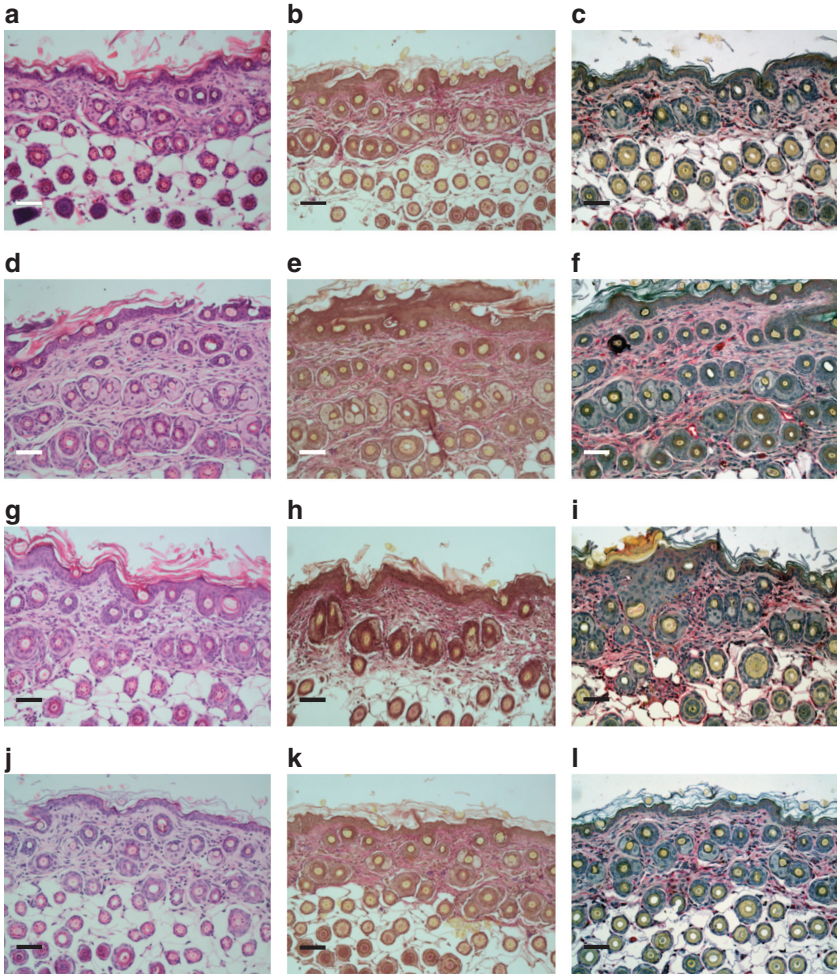


Figure 7. Suppression of TGF-β1-induced skin fibrosis *in vivo*. Cutaneous fibrosis in newborn mice was induced by repetitive TGF-β1 injections. Mice were injected on three consecutive days intracutaneously in the neck with PBS/BSA (**a–c**), 800 ng TGF-β1 (**d–f**), or TGF-β1 plus 50 μM of LZNT (**g–i**) or LZNP (**j–l**). On day 4, mice were killed, and 4-mm punch biopsies were taken from the sites of injection. Paraffin-embedded biopsies were processed for hematoxylin and eosin staining (**a**, **d**, **g**, **j**), van Gieson staining (**b**, **e**, **h**, **k**) in which collagen fibers appear red, and immunohistochemistry using an antibody against collagen type I (**c**, **f**, **i**, **l**), in which bound antibodies were visualized by the immunoperoxidase technique (red, immunoreactivity). Bar = 50 μm. Magnification, × 200.

Table 2. Antifibrotic effect of DP IV inhibitors in a mouse model of cutaneous fibrosis

	PBS	T	T+LZNP	T+LZNT
Dermal thickness (μm)	191 ± 26^a	241 ± 57	202 ± 33^a	213 ± 34^b
α-SMA expression	9 ± 6^a	30 ± 7	23 ± 6^b	22 ± 9^b

DP IV, dipeptidyl peptidase IV; LZNP, Lys[Z(NO_2)]-pyrrolidide; LZNT, Lys[Z(NO_2)]-thiazolidide; PBS, phosphate-buffered saline; α-SMA, α-smooth muscle actin; T, TGF-β_1.
$^aP<0.001$ *versus* T; $^bP<0.01$ *versus* T.
The antifibrotic effect of LZNP and LZNT (50 μM) injected with TGF-β1 (10 ng/ml) was quantified using the vertical dermal thickness as an indicator for fibrosis. The data represent the mean ± SD of 40 measurements taken from the central part of the biopsies of four different animals in each group (10 measurements per animal). The number of immunoreactive cells was determined in 4 × 4 viewing areas of deparaffinized skin sections stained for α-smooth muscle actin.
Statistics were calculated by one-way analysis of variance.

(Leask and Abraham, 2004). However, to our knowledge, in contrast to the related FAP-α (Gorrell, 2005), DP IV has never been shown to be associated with the development of fibrosis.

KFs can be considered as ''activated'' fibroblasts, as they show an increased proliferation rate and increased sensitivity toward TGF-β_1-mediated effects on collagen and matrix production as compared with NFs (Babu *et al.*, 1992; Bettinger *et al.*, 1996). As DP IV expression was shown to be upregulated upon activation in T cells and keratinocytes (Novelli *et al.*, 1996; De Meester *et al.*, 1999), we were interested to see whether DP IV expression was altered in KFs compared with NFs. We found a moderate but significantly lower DP IV-like activity and DP IV surface expression in KFs, which had no impact on inhibitor-mediated effects; the antiproliferative activity of DP IV inhibitors as well as the effects on TGF-β_1 expression and collagen and matrix biology were comparable in both cell types. However, as decreased DP IV expression has also been associated with higher invasive potential in malignant cells (Pethiyagoda *et al.*, 2000; Kikkawa *et al.*, 2005; Sato *et al.*, 2005), it might be possible that DP IV expression is functionally related to the ''invasive'' phenotype of keloids.

It has been previously shown (Lankas *et al.*, 2005) that our inhibitors LZNP and LZNT are not selective for DP IV and also inhibit the cytoplasmic DP IV-like enzymes DP8 and DP9 and, with a 100-fold less intensity, FAP-α. Although FAP-α is probably not a target of LZNP or LZNT in the concentrations used in our experiments, it can presently not be excluded that DP8 or DP9 or other yet unknown DP IV-like enzymes are involved in mediating the observed biological effects. The possible contribution of intracellular targets also provides an explanation for discrepancies between concentrations needed to suppress enzyme activity and those mediating biological effects, which are usually 5- to 10-fold higher.

The detailed and comprehensive mechanisms of how LZNT and LZNP affect fibroblast functions are yet unclear. The most relevant findings of our study are that these inhibitors significantly suppress TGF-β_1 auto-production of fibroblasts, without directly regulating mRNA expression, and that they reduce TGF-β_1-stimulated phosphorylation of phospho-p38 mitogen-activated protein kinase and pERK without affecting pSMAD3 phosphorylation. The observed decreased mRNA expression at late time points (48 hours) might also result from the activation or inactivation of other yet unidentified cofactors induced upon inhibitor incubation, or be a consequence of cell cycle arrest.

Inhibition of ERK activation leads to decreased fibroblast proliferation (Martinez-Salgado *et al.*, 2006). Therefore, the suppression of ERK phosphorylation is probably involved in mediating the antiproliferative effects of inhibitors of DP IV-like activity in fibroblasts. Interestingly, the basal inhibitor-induced suppression of pERK and proliferation is slightly more pronounced in keloids, whereas the suppression of TGF-β_1-stimulated pERK is diminished as compared with NFs. This observation suggests that ERK activation is not mainly responsible for the previously described increased proliferation of KFs toward TGF-β_1 (Bettinger *et al.*, 1996). Furthermore, p38 was shown to regulate collagen synthesis, matrix deposition, and MMP production in fibroblasts (Sato *et al.*, 2002; Munshi *et al.*, 2004; Li *et al.*, 2006), and belongs to the SMAD-independent pathway of the TGF-β_1-signalling cascade. Moreover, DP IV has been shown to mediate cell adhesion to fibronectin and collagen I through p38-MAPK-regulated phosphorylation of integrin β_1 in the Karpas 299 T-anaplastic large-cell lymphoma line (Sato *et al.*, 2005).

Thus, the suppression of p38 phosphorylation by inhibitors of DP IV-like activity provides one possible mechanism of how antifibrotic effects of these substances are mediated. Surprisingly, the inhibitor sensitivity of the p38 pathway is much increased in KFs as compared with NFs, whereas the effects on collagen and matrix metabolism are comparable in both cell types, which suggests an indirect or complex relationship. If p38 contributes to increased collagen production of keloids, the inherent inhibitor sensitivity of this pathway would be helpful for therapy by targeting the altered cells more selectively.

A special characteristic of keloid invasiveness is an increased migratory activity associated with higher MMP-1 (interstitial collagenase) and MMP-2 (gelatinase-A) production (Fujiwara *et al.*, 2005). TGF-β_1 is known to induce expression of MMP-2, MMP-9, and MMP-13 in fibroblasts (Ravanti *et al.*, 1999; Saed *et al.*, 2000), whereas MMP-1 expression is negatively regulated through SMAD3 and 4 (Yuan and Varga, 2001). Using an antibody array with subsequent semi-quantitative analysis, we have largely reproduced these stimulating effects of TGF-β_1 on MMP-2 and MMP-13 in both KFs and NFs and, in addition, found a previously unknown stimulatory activity on MMP-8 expression in both cell types. In the presence of inhibitors of DP IV-like activity, the stimulatory effect of TGF-β_1 on MMP-2 and MMP-13 expression was diminished, whereas DP IV inhibitors alone slightly reduced MMP-1 and MMP-2 expression. This might have an impact on keloid migratory activity, which has to be further substantiated. The decreased MMP expression suggests that reduced collagen synthesis rather

than increased collagenolysis mediates the suppression of collagen deposition in the presence of inhibitors. Although the results of the array have to be interpreted with caution and further confirmed by other quantitative methods, these data suggest that the inhibitor-related effects on fibroblast functions are mediated by a variety of mechanisms and cofactors.

Summarizing, the antifibrogenic activity of inhibitors of DP IV-like activity *in vitro* and *in vivo* adds another dimension to the broad spectrum of possible therapeutic applications of this promising substance group. Further investigations will have to clarify the differential contribution of other DP IV-like proteins to the observed biological effects and the detailed mechanisms to provide a rationale for improved molecule- and disease-targeted inhibitor design. It might be advantageous for these substances that inhibition of DP IV-like activity interacts only partly with TGF-β_1 signalling and that suppression of TGF-β_1 production has so far only been observed in fibroblasts, but not in other immune or epithelial cells. Broad suppression of this pluripotent and ubiquitously distributed cytokine could be potentially hazardous if the various physiological roles of TGF-β_1 are considered, for example, immunosuppressive, anti-inflammatory, tumor-suppressive, and antiproliferative effects.

MATERIALS AND METHODS

All described studies using materials obtained from human subjects were conducted according to the Declaration of Helsinki Principles and approved from the medical ethical committee of the Otto-von-Guericke University, Magdeburg. Participants gave their written informed consent.

Sources of fibroblasts

Normal human skin fibroblasts were cultured from discarded tissue of dermatomed skin from the trunk or thigh of adults of Caucasian race, through explant culture. Keloids were collected from six patients of Caucasian origin aged 15–67 years (mean 36.6 ± 20 years, median 35.4 years) who had undergone keloid excision in the Dermatology Clinic of the Otto-von-Guericke University Magdeburg, after they had given their written informed consent. All keloids included in this study were confirmed by histologic examination. The keloids were obtained from the trunk ($n = 3$), arm ($n = 2$), and ear lobe ($n = 1$), and grown through explant culture using the second to sixth passages for the experiments.

Cell culture conditions

Both NFs and KFs were routinely cultured in Iscove's Medium supplemented with 1% penicillin/streptomycin and 10% fetal calf serum (FCS) (all from Biochrom, Berlin, Germany) in a humidified atmosphere of 5% CO_2 at 37°C.

For inhibitor experiments, cells were either grown in serum-free Fibromed-Medium or DMEM, with 3.7 g/l $NaHCO_3$, 4.5 g/l D-glucose, and glutamine supplemented with 0.1% FCS (both from Biochrom). LZNP and LZNT were synthesized by K Neubert and J Faust. Cell viability was determined using the lactate dehydrogenase cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) at time points of 4 and 6 hours after inhibitor incubation and by Trypan blue (Sigma-Aldrich, Deisenhofen, Germany) staining.

Immunofluorescence staining of cells

Immunofluorescence staining of fibroblasts cells was performed using the monoclonal anti-CD26 antibody clone M-A261 (mIgG1; BD Biosciences, Heidelberg, Germany). For control of nonspecific labelling, cells were incubated with an irrelevant IgG1 monoclonal mouse antibody (BD Biosciences). Labelled cells were analyzed by flow cytometry (FACS-Calibur; BD Biosciences).

Enzymatic assay

DP IV-like activity was determined in triplicate in PBS, pH 7.4, using Gly–Pro–4-nitroanilide (Sigma-Aldrich) as chromogenic substrate. A 100-µl volume Gly–Pro–4-nitroanilide was added in a concentration of 3 mM to cellular suspensions of 10^5 cells/100 µl. Reactions were stopped by adding 900 µl acetate buffer (1 M pH 4.4) either immediately (control) or after 120 minutes of incubation at 37°C. After centrifugation (2 minutes, 10,000 × *g*), the absorbance of the supernatant was detected spectrophometrically at 392 nm.

RNA preparation from NFs and KFs and qualitative RNA amplification

The RNA preparation was performed as previously described (Thielitz *et al.*, 2007). A 1.2-µl volume of the reverse transcription reaction was used as template for amplification. Forty cycles were performed in a Peltier thermal cycler PTC-200 (Biozym Diagnostik, Hessisch Oldendorf, Germany) in 50 µl reaction buffer containing 2.5 U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), 200 µM dNTP, and 0.5 µM of the corresponding primer set of DP IV ((5′–3′) sense GATGCTACA GCTGACAGTCGC, (5′–3′) antisense TGGTGACCATGTGACCCACTG); DP8 ((5′–3′) sense AGATCAACCT GACCTGGCC, (5′–3′) antisense ACGGAAGCAGGATCTTCTGG); DP9 ((5′–3′) sense TCCAGGTGCAGAAGCACTCG, (5′–3′) antisense TAGGGGACACCATGAAGCCG; and FAP-α ((5′–3′) sense CAAAGA CCAGGAGATCCACC, (5′–3′) antisense AACAAAGAATCCACCAG CCC) obtained from BioTeZ (Berlin, Germany). The initial activation step at 95°C for 15 minutes was followed by 40 cycles of denaturation for 1 minute at 94°C, annealing for 0.5 minutes at 60°C, and extension at 72°C for 1 minute. The reaction was stopped after a final extension step at 72°C for 5 minutes and products were visualized on a 1.5% agarose gel.

Proliferation assay

NFs and KFs were seeded in a concentration of 2×10^5 cells/ml into 96-well plates (PAA, Cölbe, Germany) in serum-free medium. Different concentrations of the inhibitors (3.125–50 µM) LZNT and LZNP were added after 24 hours to the adherent cells. After 48 hours the cultures were pulsed for additional 6 hours with [^{3}H]methylthymidine ([^{3}H]dThd, 0.2 µCi per well; ICN, Meckenheim, Germany). The incorporated radioactivity was harvested on glass-fiber filters and measured by scintillation counting.

TGF-β_1 and fibronectin ELISAs

For the generation of supernatants, fibroblasts (NFs and KFs) in a concentration of 10^5 cells/ml were seeded in 24-well plates in culture medium containing 10% FCS. After 24 hours the medium was exchanged by low-serum medium (DMEM supplemented with 0.1% FCS), and the inhibitors, LZNT or LZNP, in concentrations of 50 µM, TGF-β_1 (10 ng/ml), or both, were added. Supernatants were collected 72 hours after the substances were added. TGF-β_1 was

measured using a commercially available ELISA kit (R&D Systems, Wiesbaden, Germany). For the analysis of fibronectin, samples were diluted 40-fold and also measured by a commercially available ELISA kit (TaKaRa, Shiga, Japan).

Determination of PICP

The amounts of PICP used as a marker for procollagen I secretion were determined using a commercially available ELISA (TaKaRa). Fibroblasts (NFs and KFs) were seeded into 12-well tissue culture plates at a density of 200,000 cells per well. After 24 hours the medium was exchanged by low-serum medium (DMEM supplemented with 0.1% FCS and 100 μg/ml L-ascorbic acid). Fibroblasts were subsequently stimulated with TGF-β_1 (10 ng/ml), the DPIV inhibitors LZNT or LZNT at a concentration of 50 μM, or both agents. Culture supernatants were harvested after 48 hours, centrifuged, and frozen at −70°C until use.

Determination of collagen and ECM deposition

Collagen secretion and deposition into ECM were assessed by a proline incorporation assay described in detail earlier (Peterkofsky and Diegelmann, 1971). All assays were performed in triplicate. Briefly, 5×10^4 fibroblasts were seeded into 24-well plates (PAA) in culture medium containing 10% FCS. After 16 hours the medium was exchanged by low-serum medium (DMEM supplemented with 0.1% FCS, 100 μg/ml L-ascorbic acid) containing [2,3,4,5-^{3}H]L-proline (2 μCi/ml; NEN, Boston, MA) and the inhibitors LZNT or LZNP (50 μM), TGF-β1 (10 ng/ml), or both, were added. After 72 hours the culture medium was removed and remaining fibroblasts were lysed with deionized water (10 minutes, room temperature). ECM was ethanol-fixed (70% ethanol, 15 minutes, room temperature). One half of the wells was incubated with 30 U/ml of collagenase (*Clostridium histolyticum*; Sigma, Deisenhofen, Germany) in collagenase assay buffer (50 mmol/l Tris-HCl, pH 7.5, 5 mmol/l $CaCl_2$, 2.5 mmol/l N-ethylmaleimide) for 4 hours at 37°C. The remaining wells were incubated with assay buffer. The supernatants were removed and residual ECM was solubilized by overnight incubation in 0.3 mol/l of NaOH–1% SDS. Equal aliquots of supernatants after collagenase digestion and supernatants containing the residual ECM were subjected to liquid scintillation counting. The counts measured in supernatants after collagenase treatment represent the collagen content. [^{3}H]Proline measured after solubilization of the remaining ECM represents non-collagenous ECM. The total of both counts was equal to the counts from solubilized ECM without collagenase treatment and represents the total proline incorporation. Relative ECM synthesis can be calculated by the established formula (Agelli and Wahl, 1988): ECM = c.p.m. in collagen + 5.4 × c.p.m. in non-collagen ECM. The formula contains the factor 5.4 to correct the 5.4-fold higher proline or hydroxyproline content of collagens compared with that of other proteins.

MMP antibody array

Supernatants were prepared as described above for the TGF-β_1 ELISA and collected 72 hours after the inhibitors LZNT or LZNP (50 μM), TGF-β_1 (10 ng/ml), or both, were added. They were analyzed by a human MMP antibody array (RayBiotech; Norcross, GA) according to the manufacturer's instructions.

Semi-quantitative analysis was performed using the software Kodak D1 3.6, by calculating three times for each dot the product of size and densitometric intensity after the separate membranes were normalized to each other using the results for the six positive controls. The results for each MMP dot are demonstrated relative to the control membrane (cells without inhibitors or TGF-β_1).

Western blotting

Fibroblasts were seeded in a density of 1×10^5/well and grown for 24 hours in low-serum medium (DMEM supplemented with 0.1% FCS), which was exchanged by serum-free DMEM 2 hours before the experiment was started. Cells were lysed in lysis buffer containing 1% NP-40, 1% laurylmaltoside (N-dodecyl b-D-maltoside), 50 mM Tris pH 7.5, 140 mM NaCl, 10 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4. Cell extracts were adjusted to represent the same amount of cellular protein (50 μg), and proteins were separated by SDS–PAGE, electrotransferred to polyvinylidene difluoride membranes, and blotted with the following antibodies: rabbit anti-phospho-p44/42-MAPK, rabbit anti-phospho-p38 MAPK, β-actin antibody (all from Cell Signaling, Beverly, MA). After three washings with Tris-buffered Saline Tween 20 (TBST), a secondary antibody, conjugated with horseradish peroxidase, was applied and the signals detected by chemoluminescence reagents (Pierce Biotechnology, Rockford, IL).

Mouse model for cutaneous fibrosis

A mouse model for TGF-β_1-induced cutaneous fibrosis described previously (Shinozaki *et al.*, 1997) was used for *in vivo* evaluation of the antifibrogenic effect of DP IV inhibitors. The rationale to use this model was the background that increased TGF-β_1 production is involved in the pathogenesis of keloids and other fibrotic conditions. Accordingly, cutaneous fibrosis was induced by intracutaneous injections of 800 ng of TGF-β_1 into the neck of newborn Balb/c mice on three consecutive days. Treatment groups (four groups of three mice each) consisted of mice injected with TGF-β_1, TGF-β_1 plus LZNP or LZNT (50 μM), and the solvent (0.1% BSA in PBS) in which TGF-β_1 had been solubilized. On day 4, mice were killed and 4-mm punch biopsies were taken from the sites of injection for (immuno-)histochemical analysis.

Immunohistochemistry

After fixation in 4% paraformaldehyde and embedment in paraffin, biopsies from mouse skin were processed with the following stains: (1) hematoxylin and eosin and (2) van Gieson stain, in which collagen appears red; (3) for collagen staining, deparaffinized sections were incubated with proteinase K solution (DakoCytomation GmbH, Hamburg, Germany) for 1 minute, washed three times in Tris-buffered saline solution containing 0.05% Tween 20, pH 7.6, and consecutively stained with a rabbit antibody against collagen type I (1:500; DPC Biermann, Bad Nauheim, Germany) for 1 hour (4) for α-smooth muscle actin staining, sections were microwave treated to unmask epitopes, followed by incubation with a polyclonal antibody from Abcam (Cambridge, UK) for 30 minutes. Negative controls included incubation with control IgG at the same protein concentration as the primary antibody, and omission of the primary antibody.

The consecutive steps were performed using standard immunoperoxidase techniques following the protocol of the LSAB-2 System-AP staining kit (DakoCytomation). Slides were incubated for 20 minutes in the dark with the chromogenic substrate Fast RED,

rinsed with, and placed for 10 minutes in water, and finally counterstained with Mayer's hematoxylin (10 seconds) before mounting in Faramount Aequous Mounting Medium (DakoCytomation).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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