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PKC412 induces apoptosis through a caspase-dependent mechanism in human keloid-derived fibroblasts

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Abstract

There is no established pharmacological therapy for skin keloids, a wound healing disorder. In this study, we investigated the effect of *N*-benzoyl staurosporine (PKC412), a protein kinase C inhibitor, on human keloid-derived fibroblasts to examine whether this agent is applicable for the treatment of keloid formation. Although PKC412 induced apoptosis in keloid fibroblasts in a time- and dose-dependent manner, the effective concentration of this agent was much higher than that of staurosporine. Western blotting showed that both PKC412 (10 μM) and staurosporine (100 nM) cleaved pro-caspase-3 to active forms. An in vitro caspase assay also showed that PKC412 and staurosporine elevated caspase-3 activities. Carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), a caspase inhibitor with a broad spectrum, inhibited caspase-3 activities stimulated by PKC412 and staurosporine; however, only PKC412-induced apoptosis, but not staurosporine-induced apoptosis, was prevented by Z-VAD-FMK. These results suggested that PKC412-induced apoptosis, but not staurosporine-induced apoptosis, is mainly mediated by the caspase-dependent mechanism.

Keywords: Keloid; Apoptosis; PKC412; Staurosporine; Caspase

1. Introduction

Skin keloids are often considered as benign tumors. They grow extensively beyond the margin of the original wound and do not regress spontaneously. Blacks and Asians are more susceptible to hypertrophic scarring and keloid formation than Caucasians (Alhady and Sivanantharajah, 1969). Keloid lesions are formed due to disturbance in the wound healing process. The normal wound healing process consists of three phases: inflammation, proliferation, and remodeling. In keloid lesions, the developing scar remains in the proliferative phase and does not enter the remodeling phase (Murray et al., 1981; Ladin et al., 1995). In the late phase of wound healing, both cellularity and the extracellular matrix in granulation tissue decrease to form a mature scar. It has been suggested that apoptosis is a mechanism mediating the transition from granulation tissue to scar (Desmouliere et al., 1995).

Staurosporine, originally isolated from *Streptomyces* as a potential anti-fungal agent, is known to be a strong inhibitor of protein kinase C (PKC) but also causes the non-specific inhibition of several other protein kinases. To avoid non-specific effects and to develop a clinically applicable anti-tumor agent, staurosporine was chemically modified to generate analogues including PKC412. PKC412 is a selective inhibitor of the conventional PKC isoforms PKC-α, PKC-β, and PKC-γ and shows fewer adverse effects (Gescher, 1998; Fabbro et al., 1999; Fabbro et al., 2000).

In this study, we investigated whether PKC412 induces apoptotic cell death in keloid fibroblasts. Both PKC412 and staurosporine induced apoptosis and activated caspase-3 in keloid fibroblasts. Interestingly, pretreatment with Z-VAD-

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FMK, a broad-spectrum caspase inhibitor, inhibited apoptosis induced by PKC412, whereas it had no effect on staurosporine-induced apoptosis, suggesting that PKC412, but not staurosporine, induces apoptosis mainly though a caspase-dependent pathway.

2. Materials and Methods

2.1. Cell culture

Keloid tissues were obtained from two Japanese female patients by surgical excision after obtaining informed consent. The primary culture of keloid fibroblasts was performed as previously described (Arakawa et al., 1990). Keloid fibroblasts and a normal fibroblast cell line, NB1RGB, were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G (Meiji-Seika, Tokyo, Japan), and 100 μg/ml streptomycin (Meiji-Seika). The keloid fibroblasts in passages 2–8 were used for all experiments.

2.2. Stimulants

Cells were placed on plastic tissue culture dishes. After incubation for 24 h, they were treated with staurosporine ($0–10^3$ nM; Wako, Osaka, Japan) or PKC412 ($0–10^4$ nM) in serum-free medium for the periods indicated. Pretreatment with carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK, 100 μM; MBL, Nagoya, Japan) or the vehicle was carried out for 30 min before stimulation. PKC412 was kindly provided by Novartis Pharma (Basel, Switzerland).

2.3. Nuclear staining

Cells were incubated with or without stimulants for the periods indicated and then harvested by trypsin/EDTA treatment. To assess nuclear morphology, cells were stained

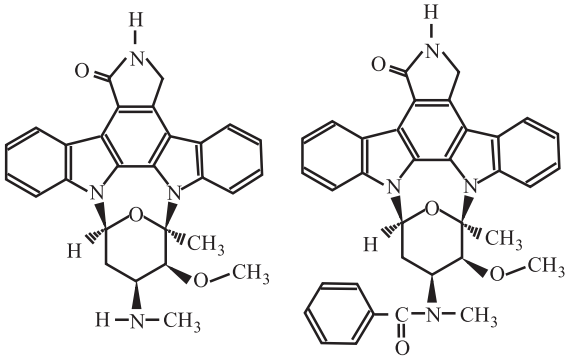

Fig. 1. Structures of staurosporine and PKC412.

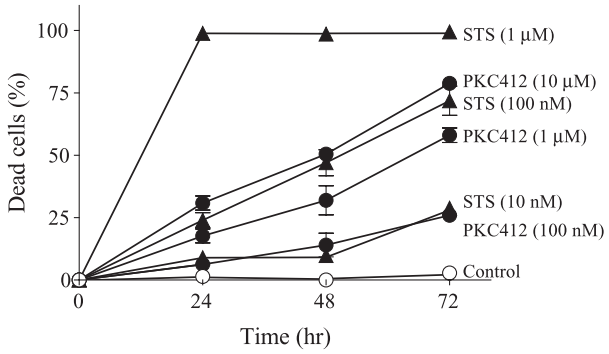

Fig. 2. PKC412 and staurosporine induced apoptosis in keloid fibroblasts. Keloid fibroblasts were treated with or without various concentrations of PKC412 or staurosporine for the indicated periods (0, 24, 48, 72 h) and stained with Hoechst 33342 dye. Values are the means±S.E.M. of six independent experiments.

with 1 μM of Hoechst 33342 dye (Molecular Probes, Eugene, OR, USA) and observed under a fluorescence microscope (Olympus, Tokyo, Japan). More than 200 cells were counted for each assessment.

2.4. Detection of DNA ladder formation

Both detached and adherent cells were collected and lysed in 100 μl of lysis buffer containing 10 mM Tris/HCl (pH7.4), 10 mM EDTA (pH8.0) and 0.5% Triton X-100 for 10 min on ice. After centrifuge at 15,000 rpm for 10 min, supernatant was collected. After 1 h incubation with 2 μl of RNase A (10 mg/ml) at 37 °C, 2 μl of proteinase K (10 mg/ml) was added and incubated at 50 °C for further 30 min. DNA was precipitated and electrophoresed using 2.0% agarose gel.

2.5. Immunoblotting

Samples were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane using a semi-dry transfer system (1 h, 15 V). After blocking with 5% skimmed milk for 30 min, the membrane was probed with a polyclonal anti-caspase-3 antibody (Upstate Biotechnology, Lake Placid, NY, USA) for 1 h at room temperature. The membrane was washed thee times and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) for 1 h. Immunoreactive proteins were then visualized by treatment with a detection reagent (LumiGLO, Cell signaling Technology, Beverly, MA, USA).

2.6. Caspase-3 activity assay

In vitro caspase-3 activity assay was carried out as previously described (Nakazono-Kusaba et al., 2002). Cells were incubated with or without stimulants for the periods indicated and lysed on ice for 10 min. The protein (20 μg)

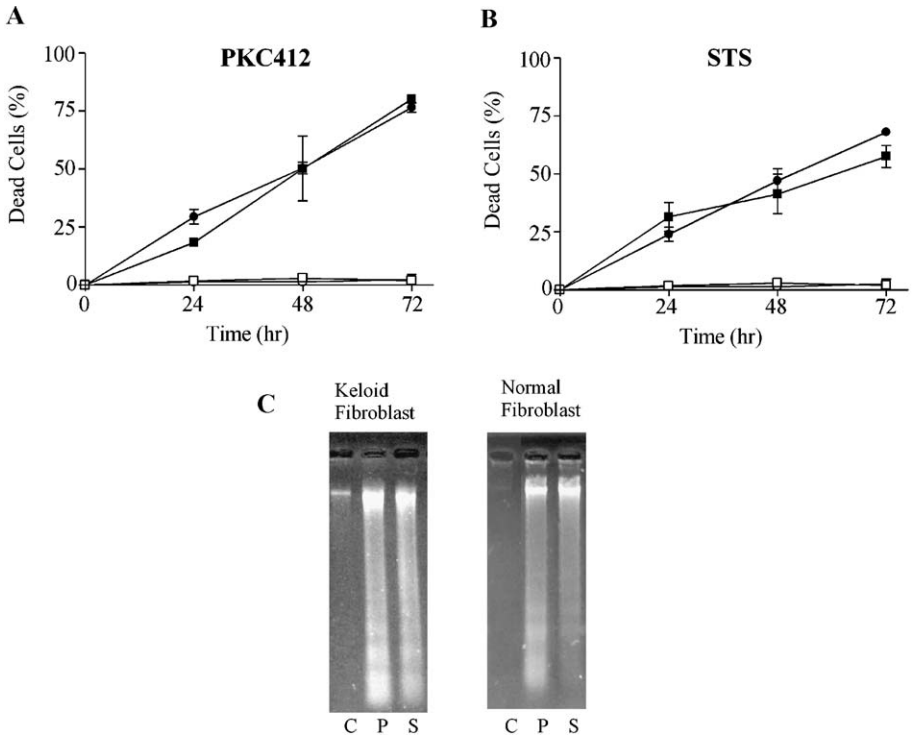

Fig. 3. Effects of PKC412 and staurosporine on keloid fibroblasts and normal fibroblasts. Keloid fibroblasts and normal fibroblasts were treated with (closed symbol) or without (open symbol) 10 μM of PKC412 (A) or 100 nM of staurosporine (B) for the indicated periods (0, 24, 48, 72 h) and stained with Hoechst 33342 dye. Values are the means±S.E.M. of six independent experiments. ●: keloid fibroblasts; ■: normal fibroblasts. (C) DNA ladder formation. Keloid fibroblasts and normal fibroblasts were treated with or without PKC412 (10 μM) or staurosporine (100 nM) for 48 h and extracted DNA was analyzed by electrophoresis. C, control; P, PKC412; S, staurosporine. Results are representative of three independent experiments.

was incubated with 200 μM Asp-Glu-Val-Asp (DEVD)-*p*-nitroanilide (Caspase-3 colorimetric protease assay kit, MBL) at 37 °C for 24 h. The absorbance of the samples was measured at 405 nm to determine the caspase-3 activity.

3. Results

3.1. Staurosporine and PKC412 induced apoptosis in keloid-derived and normal fibroblasts

PKC412 (*N*-benzoyl staurosporine) is a selective inhibitor of conventional PKCs and a potential anti-tumor agent (Fig. 1). It is reported to induce apoptosis in tumor cells and sensitize tumor cells to other cytotoxic agents (Gescher, 1998; Fabbro et al., 1999; Fabbro et al., 2000).

Since keloid lesions are considered as benign dermal tumors, the effect of PKC412 on keloid fibroblasts was tested. To examine whether PKC412 and staurosporine induce apoptosis in keloid fibroblasts, we stained the nucleus using Hoechst 33342. The nuclei of viable cells were diffusely stained, whereas apoptotic cells showed nuclear shrinkage and chromatin condensation (Kerr et al., 1972; Raff, 1992). As shown in Fig. 2, apoptotic cells were significantly increased by treatment with PKC412 and staurosporine in time- and dose-dependent manner. However, the effects of these compounds differed in strength.

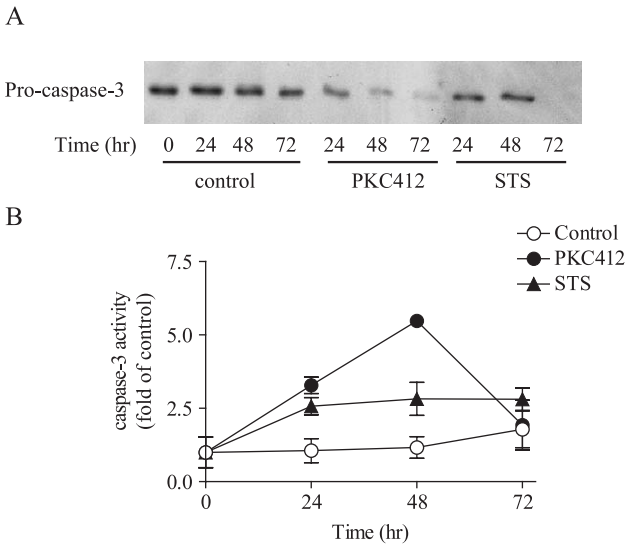

Fig. 4. PKC412 and staurosporine activated caspase-3. (A) Immunoblot analysis of casapase-3. Cells were treated with 10 μM of PKC412 or 100 nM of staurosporine for the indicated periods (0, 24, 48, 72 h). Samples were separated by 15% SDS-PAGE and immunoblot analysis was carried out using an anti-caspase-3 antibody. Results are representative of three independent experiments. (B) Time course of caspase-3 activity induced by PKC412 and staurosporine. Cells were treated with or without 10 μM of PKC412 or 100 nM of staurosporine for the indicated periods (0, 24, 48, 72 h). Cell lysate was incubated with DEVD-*p*NA at 37 °C for 3 h and the absorbance was measured at 405 nm. Values are the means±S.E.M. of three independent experiments.

Staurosporine at 100 nM had almost the same effect as 10 μM of PKC412, suggesting that PKC412 was much less potent than staurosporine in its ability to induce apoptosis. We used these concentrations (10 μM for PKC412 and 100 nM for staurosporine) for the following experiments. We could not find any inter-individual difference between donors in the concentration dependence. Therefore, we use the cells from one donor for the following experiments.

Since it has been reported that keloid fibroblasts showed resistance to apoptosis compared with normal fibroblasts in vitro (Ishihara et al., 2000; Chondon et al., 2000; Luo et al., 2001), we compared the effects of PKC412 and staurosporine between normal fibroblasts and keloid fibroblasts. As shown in Fig. 3, PKC412 (A) and staurosporine (B) similarly induced apoptosis in both normal and keloid fibroblasts, suggesting that there was no difference between normal and keloid fibroblasts in the sensitivity to PKC412 and staurosporine for the induction of apoptosis. The levels of apoptosis induced by PKC412 and staurosporine were also determined by examining DNA fragmentation. PKC412 and staurosporine similarly induced DNA ladder formation in normal and keloid fibroblasts (Fig. 3C).

3.2. PKC-412 induced apoptosis though a caspase-dependent pathway in keloid-derived fibroblasts

We examined whether PKC412 and staurosporine activate caspase-3 in keloid fibroblasts. As shown in Fig. 4A, PKC412 and staurosporine decreased the amount of pro-caspase-3 in a time-dependent manner, indicating that both agents stimulated caspase-3. An in vitro caspase-3 assay also showed that PKC412 and staurosporine stimulated caspase-3 (Fig. 4B). After 48 h stimulation, the activity of caspase-3 was significantly higher in PKC412-treated cells than staurosporine-treated cells. PKC412 (10 μM) seemed to be equivalent to staurosporine (100 nM) in its ability to induce apoptosis (Fig. 2); however, PKC412 (10 μM) activated caspase-3 stronger than staurosporine (100 nM) in keloid fibroblasts (Fig. 4A and B). This suggested that the apoptosis induced by PKC412 may be much more caspase-dependent than the apoptosis induced by staurosporine.

To confirm this hypothesis, we next examined the effects of Z-VAD-FMK, a caspase inhibitor with a broad spectrum, on caspase-3 activity and apoptosis induced by PKC412 and staurosporine. As shown in Fig. 5A, Z-VAD-FMK strongly inhibited PKC412- and staurosporine-

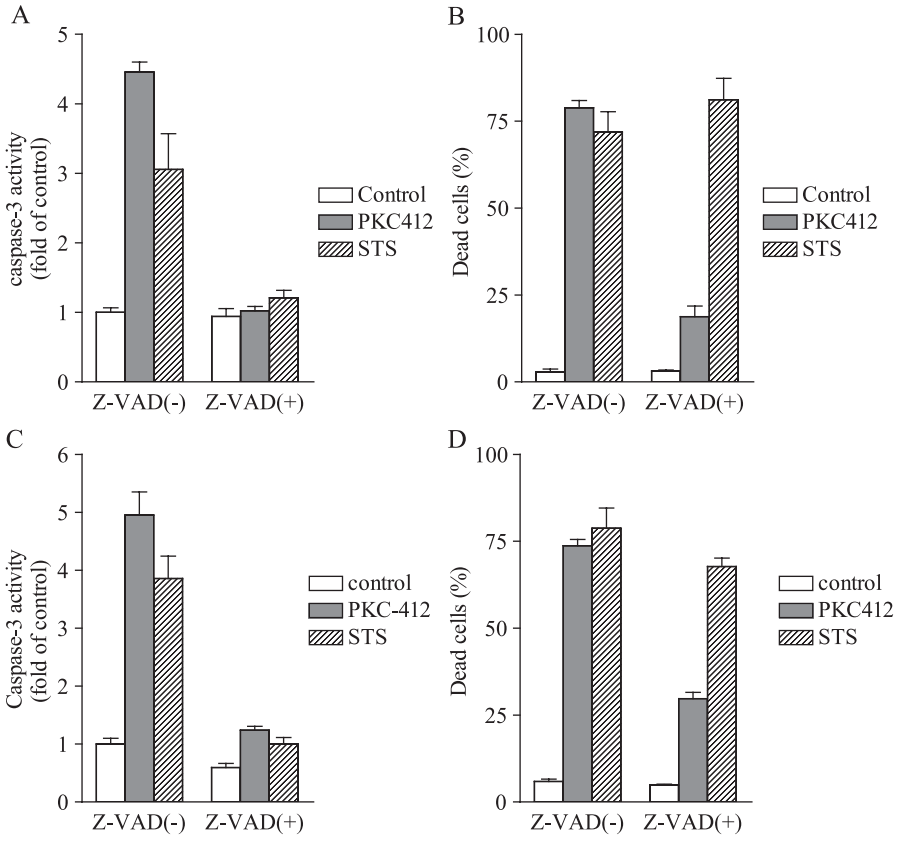

Fig. 5. Effects of Z-VAD-FMK on caspase-3 activity and apoptosis induced by PKC412 and staurosporine. (A, B) Caspase-3 activity. Keloid fibroblasts (A) and normal fibroblasts (B) were pretreated with 100 μM of Z-VAD-FMK for 30 min and stimulated with or without 10 μM of PKC412 or 100 nM of staurosporine for 48 h. Cell lysate was incubated with DEVD-*p*NA at 37 °C for 3 h and the absorbance was measured at 405 nm. Values are the means±S.E.M. of three independent experiments. (C, D) Apoptosis. Keloid fibroblasts (C) and normal fibroblasts (D) were pretreated with 100 μM of Z-VAD-FMK for 30 min and stimulated with or without 10 μM of PKC412 or 100 nM of staurosporine for 72 h. Cells were harvested and stained with Hoechst 33342 dye. Values are the means±S.E.M. of three independent experiments.

induced caspase-3 activities. Z-VAD-FMK also inhibited PKC412-induced apoptosis. However, strikingly, Z-VAD-FMK had no effect on staurosporine-induced apoptosis (Fig. 5C). This result suggested that, at least in keloid fibroblasts, PKC412-induced apoptosis is largely caspase-dependent, whereas a large part of staurosporine-induced apoptosis is caspase-independent. Next, we examined the effect of Z-VAD-FMK on staurosporine- and PKC412-induced caspase-3 activity and apoptosis in normal fibroblasts. As shown in Fig. 5B, Z-VAD-FMK inhibited caspase-3 activity induced by PKC412 and staurosporine in normal fibroblasts. On the other hand, this compound inhibited PKC412-induced apoptosis, whereas it had no effect on staurosporine-induced apoptosis (Fig. 5D). These results indicated that, not only in keloid fibroblasts but also in normal fibroblasts, PKC412-induced apoptosis is largely caspase-dependent.

4. Discussion

It has been reported that keloid fibroblasts are resistant to apoptosis induced by several stimuli, i.e., C2 ceramide, serum starvation, irradiation, anti-Fas antibody, compared with normal fibroblasts in vitro (Ishihara et al., 2000; Chondon et al., 2000; Luo et al., 2001). Chondon et al. (2000) reported that keloid fibroblasts are less affected by staurosporine. In this study, however, there was no significant difference in the sensitivity to apoptosis induced by PKC-412 and staurosporine between normal and keloid fibroblasts. This discrepancy may be due to the different concentrations of staurosporine; they used 10 nM, whereas we used 100 nM.

As shown in Fig. 1, the structural difference between PKC412 and staurosporine is quite small; whether or not the amine in the glycoside ring of staurosporine is benzoylated. This small chemical modification not only made PKC412 more specific to PKC but also decreased the ability to inhibit PKC 10 times less than staurosporine (Gescher, 1998; Fabbro et al., 1999, 2000). We showed that the ability of PKC412 to induce apoptosis was 100 times less than that of staurosporine, suggesting that some mechanisms other than PKC inhibition may be involved in apoptosis induced by PKC412. Indeed, it has been suggested that there is no direct relation between PKC inhibition and the growth arrest induced by PKC412; and mechanisms other than PKC inhibition mediate growth arrest in PKC412- and staurosporine-treated cells (Gescher, 1998).

Belmokhtar et al. (2001) reported that staurosporine induces apoptosis through both caspase-dependent and caspase-independent pathways. To determine whether staurosporine and/or PKC412 require caspase activity to induce apoptosis in keloid fibroblasts, we examined the effect of Z-VAD-FMK, a broad-spectrum caspase inhibitor. Although the caspase-3 activities induced by PKC412 and staurosporine were almost completely inhibited by pretreatment with Z-VAD-FMK, only the apoptosis induced by PKC412, but not staurosporine, was inhibited by pretreatment with Z-VAD-FMK. These results suggested that both staurosporine and PKC412 induced apoptosis in keloid fibroblasts; however, these two agents used different pathways to induce apoptosis. Rocha et al. (2000) reported that both PKC412 and staurosporine enhanced mitochondrial cytochrome *c* release, resulting in apoptosis through the activation of caspase-3. Therefore, PKC412 is likely to mainly use this pathway to induce apoptosis and staurosporine may induce apoptosis mainly via other unknown pathways.

In this study, we showed that PKC412, a selective PKC inhibitor and a candidate for novel antitumor agents, induced keloid fibroblast apoptosis through a caspase-dependent pathway. As PKC412 seemed to be more specific to the caspase-dependent pathway than staurosporine, this agent may have fewer adverse effects when applied to keloid lesions.

Acknowledgments

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References

Alhady, S., Sivanantharajah, K., 1969. Keloids in various races. Plast. Reconstr. Surg. 44, 564–566.

Arakawa, M., Hatamochi, A., Takeda, K., Ueki, H., 1990. Increased collagen synthesis accompanying elevated m-RNA levels in cultured Werner's syndrome fibroblasts. J. Invest. Dermatol. 94, 187–190.

Belmokhtar, C.A., Hillion, J., Ségal-Bendirdjian, E., 2001. Staurosporine induces apoptosis though both caspase-dependent and caspase-independent mechanisms. Oncogene 20, 3354–3362.

Chondon, T., Sugihara, T., Igawa, H., Funayama, E., Furukawa, H., 2000. Keloid-derived fibroblasts are refractory to Fas-mediated apoptosis and neutralization of autocrine transforming growth factor-β1 can abrogate this resistance. Am. J. Pathol. 157, 1661–1669.

Desmouliere, A., Redard, M., Darby, I., Gabbiani, G., 1995. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. Am. J. Pathol. 146, 56–66.

Fabbro, D., Buchdunger, E., Wood, J., Mestan, J., Hofmann, F., Ferrari, S., Mett, H., O'Reilly, T., Meyer, T., 1999. Inhibitors of protein kinases: CGP 41251, a protein kinase inhibitor with potential as anticancer agent. Pharmacol. Ther. 82, 293–301.

Fabbro, D., Ruetz, S., Bodis, S., Pruschy, M., Csermak, K., Man, A., Campochiaro, P., Wood, J., O'Reilly, T., Meyer, T., 2000. PKC412-a protein kinase inhibitor with a broad therapeutic potential. Anti-cancer Drug Des. 15, 17–28.

Gescher, A., 1998. Analogs of staurosuporine: potential anticancer drugs? Gen. Pharmacol. 31, 721–728.

Ishihara, H., Yoshimoto, H., Fujioka, M., Murakami, R., Hirano, A., Fujii, T., Ohsturu, A., Namba, H., Yamashita, S., 2000. Keloid fibroblasts resist ceramide-induced apoptosis by overexpression of insulin-like growth factor I receptor. J. Invest. Dermatol. 115, 1065–1071.

Kerr, J.F., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26, 239–257.

Ladin, D.A., Garner, W.L., Smith, D.J., 1995. Excessive scarring as consequence of healing. Wound Repair Regen. 3, 6–14.

Luo, S., Benathan, M., Raffoul, W., Panizzon, R.G., Egloff, D.V., 2001. Abnormal balance between proliferation and apoptotic cell death in fibroblasts derived from keloid lesions. Plast. Reconstr. Surg. 107, 87–96.

Murray, J.C., Pollack, S.V., Pinnelli, S.R., 1981. Keliods: a review. J. Am. Acad. Dermatol. 4, 461–470.

Nakazono-Kusaba, A., Takahashi-Yanaga, F., Morimoto, S., Furue, M., Sasaguri, T., 2002. Staurosporine-induced cleavage of α-smooth muscle actin during myofibroblasts apoptosis. J. Invest. Dermatol. 119, 1008–1013.

Raff, M.C., 1992. Social controls on cell survival and cell death. Nature 356, 397–400.

Rocha, S., Soengas, M.S., Lowe, S.W., Glanzmann, C., Fabbro, D., Winterhalter, K., Bodis, S., Pruschy, M., 2000. Protein kinase C inhibitor and irradiation-induced apoptosis: relevance of the cytochrome *c*-mediated caspase-9 death pathway. Cell Growth Differ. 11, 491–499.