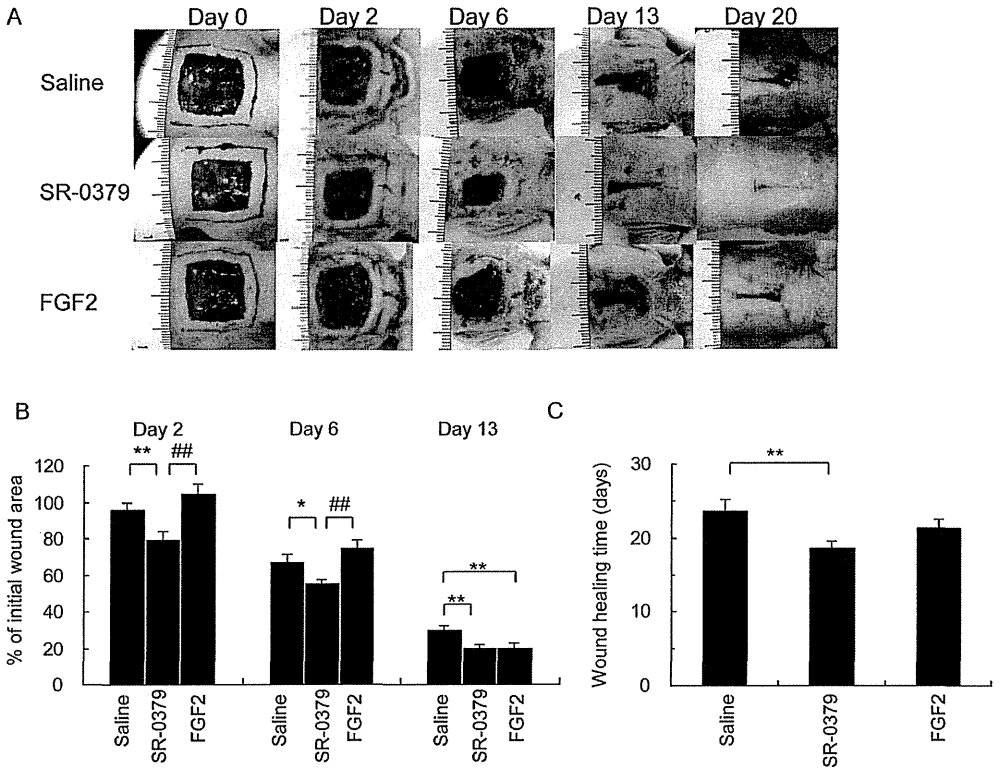

Figure 4. Effects of SR-0379 and FGF2 on full-thickness wound model with flap in diabetic rat model. A) Representative pictures of skin flaps in the streptozotocin-induced diabetic model in the saline (control), SR-0379 (0.2 mg/ml) and FGF2 groups (0.06 mg/ml) on days 0, 6, 13 and 20. B) Quantification of the wound area is represented as a percentage of the initial wound area. N = 6 per group. **P<0.01 vs. control, ##P<0.01 vs. FGF2. C) Days to complete healing by the contraction of full-thickness skin flaps in the streptozotocin-induced diabetic model.
doi:10.1371/journal.pone.0092597.g004

stimulate human umbilical vein endothelial cells (HUVECs) proliferation and tube formation at a level similar to AG30/5C. This smaller peptide led to a reduced cost of peptide synthesis. In the previous reports, the replacement with the D-form amino acid improved the proteolytic resistance of antimicrobial peptides [18]. For example, the D-amino acid variants of host defense peptide chicken cathelicidin-2 showed enhanced stability in human serum, and fully resistant to proteolysis by trypsin and bacterial proteases. The modifications increase the stability and lower cytotoxicity of the peptides without altering their antimicrobial potency. We also confirmed the degradation by the peptide bond cleavages in N-terminus of SR-0379, and the change from L-lysine to D-lysine (SR-0379) increased the resistance to serum. Importantly, SR-0379 displayed broader antibacterial activity than the original AG30 and SR-0007. The bactericidal action of antimicrobial peptides such as pexiganan is thought to result from irreversible membrane-disruptive damage [19] [20] [21]. Especially, from the mechanisms of antibacterial activity, SR-0379 exhibited the same MIC against drug-resistant strains, such as aminoglycoside-, carbapenem- and fluoroquinolone-resistant *P. aeruginosa* and MRSA and the multidrug-resistant *A. baumannii*. SR-0379 might be useful to prevent infection by these drug-resistant bacteria.

In vitro experiments with SR-0379 demonstrated the induction of proliferation, tube formation, migration and contraction. The closure of cutaneous wounds involves three processes: epithelization, connective-tissue deposition and contraction. In particular, contraction is one of the main factors contributing to epidermal wound healing [22]. The fibroblast-collagen matrix contraction model provides a unique way to study mechanisms. Treatment with SR-0379 promoted contraction in this model, which corresponds to wound healing. The stimulatory effect of SR-0379 on the wound healing process was also confirmed by two *in vivo* wound-healing models. Furthermore, SR-0379 was able to induce angiogenesis and granulation tissue formation in the paper disc model and collagen production and proliferation in the incised wound rat model. These results support the potential use of SR-0379 in the wound-healing process. The ulcer model with infection is a unique model that is especially close to a clinical situation. Importantly, SR-0379 treatment resulted in rapid healing without infection compared to FGF2.

Although the multiple functions of antimicrobial peptides are well known, the mechanisms are still unclear. For example, LL-37 is often reported in the analysis of FPR2 (formerly known as FRPL1), the promiscuous Pertussis Toxin (PTX)-sensitive GPCR and the purinergic receptor P2X7 and in the transactivation of epidermal growth factor receptor (EGFR) [3]. The activation of EGFR in epithelial cells, endothelial cells and fibroblasts by LL-37 resulted in activation of the p38 MAPK, ERK1/2 MAPK, NFkB and PI3 kinase pathways. In contrast, although we also examined the contribution of P2X7 receptors to the effect of SR-0379, the specific antagonist of P2X7 (Brilliant Blue G) failed to inhibit the effects of SR-0379 (data not shown). SR-0379 also weakly activated EGFR. Interestingly, SR-0379 strongly activated FAK, while an integrin inhibitor (RGD peptide) blocked the Akt/

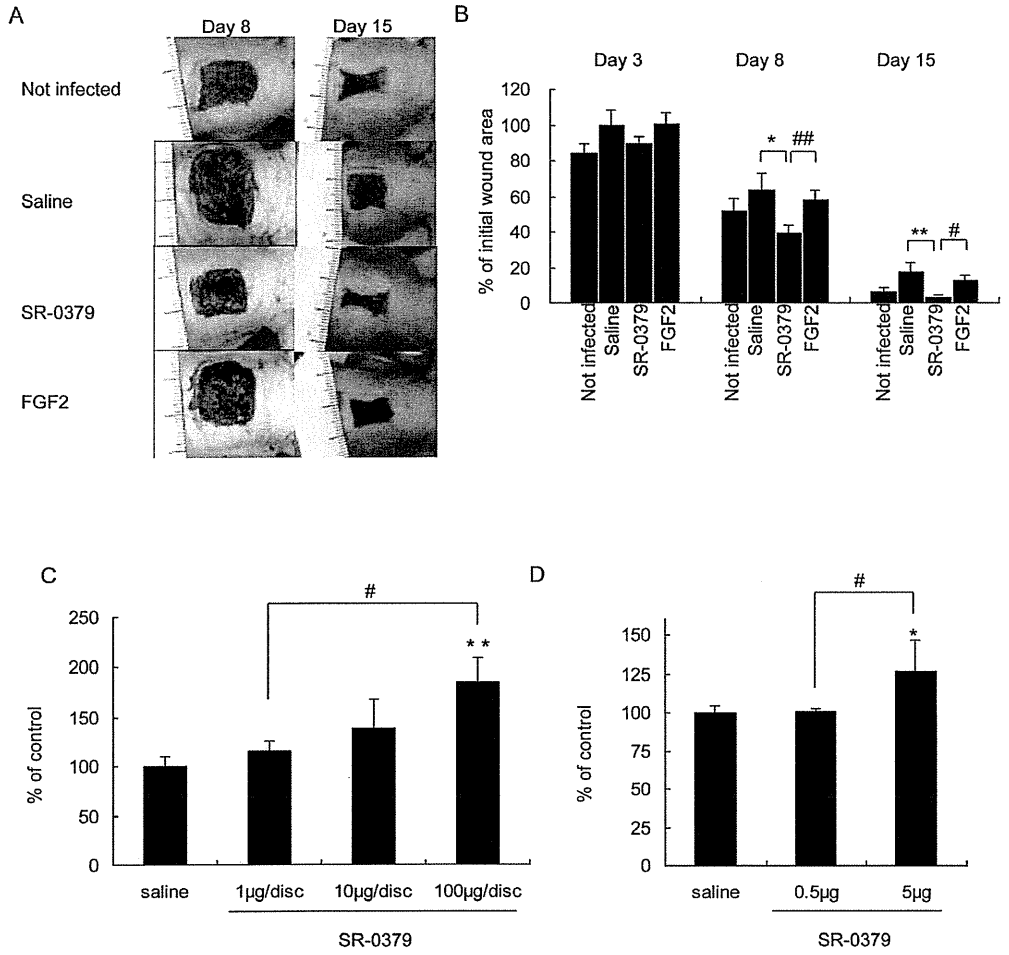

Figure 5. Effects of SR-0379 and FGF2 on the full-thickness skin infected wound model. A) Representative pictures of full-thickness skin flaps in uninfected, saline (control), SR-0379 (1 mg/ml) and FGF2 groups (0.125 mg/ml) on days 8 and 15. B) Quantification of the infected wound area is represented as a percentage of the initial wound area. N = 5 per group. **P<0.01 vs. control, ##P<0.01 vs. FGF2. C) Effects of SR-0379 (1, 10 and 100 μg/disc) on the healing of paper disc implantation in rats. N = 4-5 per group. **P<0.01 vs. control, #P<0.05 vs. 1 μg/disc. D) Effects of SR-0379 (0.5 and 5 μg) on the healing of an experimental open wound in rats. N = 3-4 per group. *P<0.05 vs. control, #P<0.05 vs. 0.5 μg.
doi:10.1371/journal.pone.0092597.g005

mTOR pathway. Downstream of FAK, SR-0379 also activated the PI3 kinase-Akt-mTOR pathway. As mTOR is known to regulate cell growth and survival by integrating nutrient and hormonal signals [10], an inhibitor, rapamycin, attenuated the proliferation induced by SR-0379 in human fibroblasts. The treatment of SR-0379 resulted in increase in cell proliferation of fibroblast, whereas Akt knockdown attenuated the SR-0379-induced cell proliferation These results demonstrate the importance of Akt pathway in the effect of SR-0379.

We have successfully produced SR-0379 as a multifunctional (angiogenic and pro-fibrotic), potent antibacterial peptide with a broad spectrum, including aerobes and anaerobes, Gram-positive and Gram-negative species and drug-resistant and drug-sensitive bacteria and fungi. These properties occur via the activation of PI3 kinase-Akt-mTOR signaling and are useful in the stimulation of wound healing under wet conditions. Further modification of SR-0379 should yield an ideal compound for the treatment of diabetic ulcers, burns and other incurable ulcers. Currently, we plan to test SR-0379 in the treatment of patients with MRSA-positive diabetic and ischemic ulcers.

Materials and Methods

Analysis of the AG30/5C metabolites using MALDI-TOF/MS

Rat sera were collected from rats. AG30/5C was incubated in pooled rat serum at 37°C. Samples were collected before incubation, after 10 minutes of incubation and after 60 minutes of incubation and were precipitated by the addition of an equivalent amount of acetonitrile containing 0.1% trifluoroacetate. The samples were centrifuged, and the supernatants were purified using ZipTip m-C18 (Millipore, MA). Sample solution was mixed with matrix solution (α-cyano 4-hydroxy cinnamic acid). The measurement sample for MALDI (0.4 μL) was applied on a MALDI target plate and dried, and the sequences of the AG30/5C metabolites were confirmed by MALDI-TOF/MS analysis (4700 Proteomics Analyzer, Applied Biosystems, CA).

Serum stability assay

In vitro stability studies were performed by incubating the peptide with rat or human serum. Human sera (Pool of donors, 5 men and 5 women) were commercially purchased from KAC (Kyoto, Japan), which has been permitted only for experiment. We don't use the human biological specimens without the documented informed consent. Rat sera were collected from rats. The peptide (500 μg/ml) was added to serum (300 μL) and incubated at 37°C. A part of samples (90 μL) were taken, and the proteins were precipitated with acetonitrile containing 0.1% trifluoroacetate (200 μL). The precipitate was separated by centrifugation. The supernatants were analyzed by high-performance liquid chromatography (HPLC).

Proliferation, tube formation, cell migration and contraction assays

HUVECs, NHDFs and NHEKs were purchased from Kurabo (Osaka, Japan). The endothelial cells were maintained in HuMedia EB2 and the fibroblasts were maintained in Medium 106S. Both media were supplemented with 1% fetal bovine serum (FBS) as described previously [9]. The epidermal keratinocytes were maintained in HuMedia KB2. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 with exchange of medium every 2 days. HUVECs were cultured in 96-well plates at a density of 10,000 cells/well and incubated for 48 hours at 37°C with AG30/5C or FGF2 (recombinant human FGF basic, R&D systems, Inc., Minneapolis, MN). The proliferation of HUVECs, NHDFs and NHEKs was analyzed using a WST-1 assay (Dojindo, Kumamoto, Japan). Tubule formation assay has recently been developed in which endothelial cells are co-cultured with fibroblasts. An angiogenesis assay kit (Kurabo, Osaka Japan) was used according to the manufacturer's instructions. Various concentrations of peptides or FGF2 were added to the medium. After 11 days, the cells were incubated with diluted primary antibody (mouse anti-human CD31, 1:4,000) for 1 hour at 37°C and diluted secondary antibody (goat anti-mouse IgG alkaline phosphatase-conjugated antibody, 1:500) for 1 hour at 37°C; visualization was achieved with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT). The tube-like structures were measured in terms of total tube length with the software (Angiogenesis Image Analyzer, Kurabo, Osaka Japan). Cell migration was evaluated using an Oris cell migration assay kit (Platypus Technologies, LLC., Madison, WI) according to the manufacturer's instructions. Briefly, the assay utilizes cell-seeding stoppers to restrict cell seeding to the outer annular regions of the wells. Removal of the stoppers reveals a 2-mm diameter unseeded region, the migration zone, into which the seeded cells migrate. The number of cells that migrated into the detection zone was measured using a plate reader. Cellular collagen gel contraction assays were performed as previously described [23] [24]. A solution of collagen and NHDFs (2×10^6 cells/ml) was added to a 24-well plate at 37°C for 1 hour, and medium supplemented with DMEM containing 10% FBS was then added. The cells were cultured for 24 hours. The culture medium was removed, and DMEM (serum-free) containing SR-0379 or FGF2 was added. The cell-embedded matrix was released from the culture dish surface. At each time point, the lattices were digitally photographed from a fixed distance, and their areas were calculated using image analysis software. In the proliferation assay of fibroblast, RGD peptide, Wotmannin, Akt inhibitor IV and Genistein were obtained from Sigma-Aldrich (St. Louis, MO). Rapamycin was obtained from Funakoshi Co., Ltd.(Tokyo, Japan). Akt siRNA I (#6211) and control siRNA I (#6568) were obtain from Cell Signaling (Boston, MA).

NHDFs were plated at a density of 5000 cells per well in 96-well culture plates in the corresponding culture media without antibiotics one day prior to transfection. Lipofectamine RNAi-MAX was purchased from Invitrogen. The lipofectamin (2 μL) was gently added to 100 μL medium and the mixture was incubated for 20 minutes at room temperature. The Akt or Control siRNA was added to the mixture and was incubated for 5 minutes. Transfection complexes were added to each well. NHDFs were incubated for 24 hours at 37°C in a CO_2 incubator and then SR-0379 (10 μg/ml) was added. NHDFs proliferation was analyzed using a WST-1 assay.

Measurement of MICs against Bacteria and Fungi

Antimicrobial activity of the peptides was evaluated against *Escherichia coli* JCM 5491, *Pseudomonas aeruginosa* JCM 6119, *Staphylococcus aureus* JCM2874, *Salmonella* Typhimurium JCM1652, *Acinetobacter baumannii* JCM6841, *Bacteroides fragilis* JCM11019, *Fusobacterium nucleatum* JCM11025, *Penicillium glabrum* JCM22534, *Fusarium solani* JCM11383, *Alternaria alternata* JCM5800 (RIKEN, A research institution for basic and applied science in Japan), *Micrococcus luteus* NBRC13867, *Bacillus subtilis* NBRC3134, *Propionibacterium acnes* NBRC107605, *Trichophyton mentagrophytes* NBRC6124, *Trichophyton rubrum* NBRC9185, *Candida krusei* NBRC1395 (National Institute of Technology and Evaluation, Tokyo, Japan), *Salmonella* Enteritidis IID604 (The Institute of Medical Science, The University of Tokyo, Tokyo, Japan). Additionally, the clinical isolates (Drug-sensitive/resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus*, Osaka University Hospital) and multidrug-resistant *Acinetobacter baumannii* (ATCC BAA-1605) were used. The MICs (expressed as μg/ml) of AG30/5C, SR-0007 and SR-0379 were determined by the broth microdilution method as previously described [8,9]. Serial two-fold dilutions of peptide were added to 0.1 ml of medium containing each type of bacteria and fungi at concentrations of 0.4×10^4 – 5×10^4 CFU/ml. The plates were incubated at 37°C with vigorous shaking for 24 or 48 hours. The MICs were determined as the lowest concentrations of peptide that inhibited visible bacterial growth.

Western blot analysis

Protein extracts (15 μg) were resolved by 10% SDS-PAGE and were then transferred to nitrocellulose membrane. Western blotting was performed. Phospho FAK (Tyr397), Akt, phospho Akt (Ser 473), mTOR, phospho mTOR (Ser2448) and α-Tubulin antibodies were obtained from Cell Signaling (Boston, MA). FAK antibody was obtained from Millipore (Billerica, MA). Phospho FAK (Tyr925) antibody was obtained from Abcam (Cambridge, MA).

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis and ELISA

Expression of the human IL-8 mRNA was measured using real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the tissue samples using ISOGEN reagent (NIPPON GENE, Toyama, Japan). Complementary DNA (cDNA) was synthesized using the Thermo Script RT-PCR System (Invitrogen, Carlsbad, CA). Relative gene-copy numbers for IL-8 mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by real-time RT-PCR using TaqMan Gene Expression Assays (IL-8: Hs00174103_m1; GAPDH: 4352934). Absolute gene-copy numbers were normalized to GAPDH using a standard curve.

The cell free culture supernatants were harvested after treatment of SR-0379 (1, 3 and 10 μg/ml) at 24, 48 and 72 hours.

The amount of IL-8 was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Effect of SR-0379 on wound healing in a streptozotocin-induced diabetic model

This experimental protocol was approved by the committee for ethics in animal studies of AnGes MG. Male HWY/Slc rats (7 weeks) were given a single intravenous injection of 65 mg/kg streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO), and whole-blood glucose was monitored 24 hours later. This strain is hair less in adult and suitable for wound healing model. The glucose level criterion for diabetes was set at 300 mg/dl. STZ-induced diabetic rats were anesthetized. The square flap (1.73 cm × 1.73 cm) was made in the back of rats. In the center of the flap, the square wound (1.41 cm × 1.41 cm) with full-thickness defect was made (area per wound; 2 cm^2). In the flap model, skin was cut in three directions of square wound to partially block the blood flow to wound. SR-0379 (0.2 mg/ml, 50 μl), FGF2 (0.06 mg/ml, 50 μl) or saline (control) was administered to each wound (each time point from day 0 to 28). Dressings (Perme-roll, Nitto Denko, Japan) were applied to the wounds. We took a picture of wound with scale every time and calculated the size of scanned image using software (http://hp.vector.co.jp/authors/VA004392/Download.htm#lenara).

Effect of SR-0379 on wound healing in a cyclophosphamide-induced immunodeficient infection model

Male HWY/Slc rats (7 weeks) were given a single intravenous injection of 100 mg/kg cyclophosphamide (CPA, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and were anesthetized for the preparation of a full-thickness skin flap 24 hours later. CPA-treated rats with white blood cell counts lower than 5,000 were used. The bacteria (*S. aureus*, 1×10^5 CFU/ml) was applied to each wound on days 0, 1, 2 and 3. SR-0379 (1 mg/ml, 50 μl), FGF2 (125 μg/ml, 50 μl) and saline (control, 50 μl) were administered to the wound at time points on days 0 to 27. Dressings (Perme-roll, Nitto Denko, Japan) were applied to the wounds. Healing size was evaluated by photographing the wound area at a close and fixed distance. The remaining unhealed wound size was measured from the image.

Evaluation of granulation tissue formation in a paper disc implantation model

Granulation tissue formation was determined as described previously [25]. A paper disc containing saline or SR-0379 (1, 10 and 100 μg) was implanted into the subcutaneous tissue on the backs of 9-week-old Crl:CD(SD) rats under anesthesia. Four or five rats were used for each experimental condition. The paper disc was removed on day 8 and the granulation tissue around the paper disc was weighed after the removal of absorbed fluids with paper wipe.

Evaluation of collagen production and proliferation in the incised wound rat model

The dermises of Crl:CD(SD) rats (7 weeks) were incised under anesthesia. In the back of rats, we cut the skin (30 mm) and sutured 3 points (Nylon thread, Natsume Seisakusho Co., Ltd., Tokyo, Japan). SR-0379 (0.5 and 5 μg per day) was topically administered in sutured wound one a day for 5 days, and during the period the suture was removed at day 3. At day 6, extracted skin was fixed in one side and pulled in another side. The tension was monitored until the opening of sutured wound. In this evaluation, the increase in tension reflects the strength of sutured wound.

Statistical analysis

All values are expressed as the means + SEM. Analysis of variance and a subsequent Fisher's Least Significant Difference test were used to determine the significance of differences in multiple comparisons.

Supporting Information

File S1 Supporting figures S1–S4. Figure S1, MALDI-TOF MS analysis. A) Major metabolites of AG30/5C determined by MALDI-TOF MS. Parent compound (AG30/5C) was incubated with rat serum *in vitro* for 10 minutes and 60 minutes. The metabolites were identified by the comparison with that from pre-incubation. Figure S2, Effect of SR-0379 on cell proliferation. Normal Human Epidermal Keratinocytes (NHEKs) were treated with SR-0379 (1, 3 and 10 μg/ml). The results were shown as percent increase compared with control (no treatment). N = 3 per group. *P<0.05 vs. control. Figure S3, Effect of Akt pathway on SR-0379-induced cell proliferation. A) Knockdown of Akt expression by siRNA was confirmed with western blot analysis anti-Akt antibody and anti-α-tubulin antibody. The sample was extracted from NHDFs with no treatment (NT), non-target siRNA (C: control) and Akt siRNA. B) Effects of Akt inhibitor on NHDFs proliferation stimulated by SR-0379. The cells were preincubated with Akt inhibitor IV (1 μM) for 1 hour and then were treated with SR-0379 (1, 3 and 10 μg/ml). N = 3 per group. *P<0.05 vs. control, **P<0.01 vs. control, ## P<0.01 vs. SR-0379 (1 μg/ml), †† P<0.01 vs. SR-0379 (3 μg/ml), ‡‡ P<0.01 vs. SR-0379 (10 μg/ml). Figure S4, Up-regulation of interleukin-8 (IL-8) induced by treatment of SR-0379. A) IL-8 mRNA expression was quantified by real time PCR and shown as a relative expression compared with that of GAPDH mRNA. NHDFs were treated with SR-0379 (10 μg/ml) for 24 hours. Effects of Wortmannin (PI3kinase inhibitor, 100 nM) and Genistein (Tyrosine-specific protein kinase inhibitor, 100 nM) on SR-0379-induced IL-8 mRNA expression. N = 3 per group. *P<0.05 vs. control, **P<0.01 vs. control, ## P<0.01 vs. SR-0379 (no inhibitor). B) IL-8 levels in culture supernatants form NHDF was measured by ELISA at 24, 48 and 72 hours after treatment. NHDFs were treated with SR-0379 (1, 3 and 10 μg/ml) for 72 hours. N = 2.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HN KT YK RM. Performed the experiments: HT HN AT YS T. Kanamori T. Kaga NT. Analyzed the data: HT AT T. Kanamori. Contributed reagents/materials/analysis tools: HT AT T. Kaga. Wrote the paper: HT HN.

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国立大学法人大阪大学大学院医学系研究科
健康発達医学寄附講座における
治験薬製造のための事前検証
（製剤化、試験法設定、安定性評価）

ナガセ医薬品株式会社
研究開発部

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1. 表題

国立大学法人大阪大学大学院医学系研究科健康発達医学寄附講座における治験薬製造のための事前検証（製剤化，試験法設定，安定性評価）

2. 目的

国立大阪大学大学院医学系研究科との請負契約書及び仕様書に基づき，SR-0379外用液（創傷治癒ペプチド）の治験薬製造のための事前検証として，製剤化（処方，製品仕様等設計），試験法の設定及び試作品の安定性評価を実施することを目的とした．

3. 実施施設

名称：ナガセ医薬品株式会社 伊丹工場

所在地：兵庫県伊丹市千僧4-323

・製剤化検討：ナガセ医薬品株式会社 研究開発部 試製室

・試験法設定：ナガセ医薬品株式会社 研究開発部及び品質管理部分析室

・安定性評価：ナガセ医薬品株式会社 研究開発部 分析室

4. 研究責任者等の名称

研究総括責任者：ナガセ医薬品株式会社 研究開発部 篠田雅充

研究責任者：ナガセ医薬品株式会社 研究開発部 井上小枝

試験責任者：ナガセ医薬品株式会社 研究開発部 鶴山貴世

試験実施者：ナガセ医薬品株式会社 研究開発部 飯田規之，坂上明美，柴田千鶴

5. 実施日

試験開始日：2014年2月10日

試験終了日：2014年3月31日

6. 製剤化検討

6.1. 処方検討

6.1.1. 実施の目的及び概要

SR-0379 外用液（以下，本品と略す）の処方設計を行う．本品は，SR-0379（以下，原薬と略す） 0.01%（0.1mg/mL），0.1%(1mg/mL)，0.3%(3mg/mL)及び1%(10mg/mL)の，4種の濃度での治験薬製造を予定している．そこで，これら4種の濃度の外用液について，製剤pHの設定検討を実施した．

本品は，事前情報として，①原薬に抗菌効果がある，②原薬は水に溶けやすく水に溶解させたpHは約3（2mg/mL）である，③本製剤は生理食塩水に溶解させて事前研究が実施されていることが，確認されている．

これらを踏まえて，以下の基本設計にて処方検討を実施した．

・原薬濃度：0.01%（0.1mg/mL），0.1%(1mg/mL)，0.3%(3mg/mL)，1%(10mg/mL)
・添加剤：塩化ナトリウム（0.9%）
・保存剤：添加しない
・pH調整：塩酸又は水酸化ナトリウムを用いる
・pH範囲：3.0～6.0付近にて検討

6.1.2. 実施内容

1）使用原料

以下の原材料を用いて検証を行った．

原料	製造業者	ロット番号
SR-0379＊	大阪大学大学院提供品	Y01011C1
塩化ナトリウム	ナカライテスク	V3H1038
0.1mol/L 塩酸	ナカライテスク	L3H7333
0.01mol/L 塩酸	ナカライテスク	L2T5368
0.1mol/L 水酸化ナトリウム	ナカライテスク	L3T9251
0.01mol/L水酸化ナトリウム	ナカライテスク	L3P8535

＊Peptide Content 82.5%

2）処方案

以下の処方にて，各pHにおける本品の安定性を評価した.

【1%製剤】

原料	検体ロット		
	Z0204	Z0205	Z0206
SR-0379※	500mg	500mg	500mg
塩化ナトリウム	450mg	450mg	450mg
0.1mol/L，0.01mol/L 塩酸	適量	適量	適量
0.1mol/L，0.01mol/L 水酸化ナトリウム	適量	適量	適量
精製水	適量	適量	適量
全量	50mL	50mL	50mL
pH	3.0	5.0	7.0

【0.1%製剤】

原料	検体ロット		
	Z0207	Z0208	Z0209
SR-0379※	50mg	50mg	50mg
塩化ナトリウム	450mg	450mg	450mg
0.1mol/L，0.01mol/L 塩酸	適量	適量	適量
0.1mol/L，0.01mol/L 水酸化ナトリウム	適量	適量	適量
精製水	適量	適量	適量
全量	50mL	50mL	50mL
pH	3.0	5.0	7.0

【0.01%製剤】

原料	検体ロット		
	Z0210	Z0211	Z0212
SR-0379※	5mg	5mg	5mg
塩化ナトリウム	450mg	450mg	450mg
0.1mol/L，0.01mol/L 塩酸	適量	適量	適量
0.1mol/L，0.01mol/L 水酸化ナトリウム	適量	適量	適量
精製水	適量	適量	適量
全量	50mL	50mL	50mL
pH	3.0	5.0	7.0

※　SR-0379は，Peptide Content(%)により含量換算する.

3）製造方法

① 飽和酢酸カリウム水溶液を用いて，相対湿度30％以下で安定な空間を作り，SR-0379を2時間以上，調湿させる．

② ①のSR-0379を2g（下記の計算により採取量を求める）秤量し、精製水に溶解させて正確に100mLとする．

秤取量＝2g×100/a

a：peptide content（％）

③ ②の溶液を正確に10mL量り取り，精製水で正確に100mLとする．

④ ③の溶液を正確に10mL量り取り，精製水で正確に100mLとする．

⑤ それぞれZ0104～Z0106:②，Z0107～Z0109:③，Z0110～Z0112:④の溶液を25mLずつ正確に投入する．これらの容器に塩化ナトリウム450mgを投入し，撹拌溶解させる．

⑥ 各検体を0.1mol/L塩酸，0.01mol/L塩酸，0.1mol/L水酸化ナトリウム，0.01mol/L水酸化ナトリウムでそれぞれpH3，5，7にpH調整を行った後，精製水にて全量50mLとする

⑦全試作品をガラス製サンプル管に入れる

4）安定性評価 保管条件

試作品各々を下記の条件に保管し，安定性の評価を行った．

保管条件	保管期間
5℃，なりゆき湿度	6日，10日
60℃，なりゆき湿度	6日，10日

5）評価項目及び試験方法

各時点において以下の試験を実施した．

評価項目	判定基準(仮)
性状	無色澄明
pH	（実測値の測定）
含量（残存率）	95％以上
類縁物質	（実測値の測定）

<u>試験方法</u>

① 性状：目視により確認

② pH：pHメーターにより測定

③ 含量：

・試料溶液の調製：

本品を試料溶液とする．

ただし，1％製剤については，試料溶解液にて10倍希釈する．

・標準溶液の調製：

約 24mg (相対湿度 30%以下の条件下で 2 時間以上調湿後) を量り，試料溶解液を加えて溶かし，正確に 20mL とする.

・試料溶解液：

0.1%トリフルオロ酢酸を含む，水／アセトニトリル混液（9：1）

・HPLC条件：

検出器：紫外吸光光度計 220nm

カラム：COSMOSIL Protein-R Packed Column 4.6×250mm

ガードカラム：COSMOSIL Protein-R Guard Column 4.6×10mm

カラム温度：40℃付近の一定温度

サンプルクーラー：5℃付近の一定温度

移動相：グラジエント

移動相 A：トリフルオロ酢酸 1mL に水 1000mL を加えて混和した液

移動相 B：トリフルオロ酢酸 1mL にアセトニトリル 1000mL を加えて混和した液

移動相の送液：移動相 A 及び移動相 B の混合比を次のように変えて濃度勾配制御する.

注入後の時間（分）	移動相 A (vol%)	移動相 B (vol%)
0 ～ 5	95	5
5 ～ 40	95 → 60	5 → 40
40 ～ 45	95	5

流量：1.2 mL/min

ニードル洗浄液：アセトニトリル／水の混液（4：1）

注入量：50μL

④ 類縁物質：

・試料溶液の調製：

本品を試料溶液とする.

ただし，1%濃度処方については，試料溶解液にて10倍希釈する.

・試料溶解液：

0.1%トリフルオロ酢酸を含む水／アセトニトリル混液（9：1）

・HPLC条件：

含量測定と同条件にて実施する

・類縁物質量算出方法：

得られたクロマトグラムより，面積百分率にて各類縁物質量を測定する

6.1.3. 実施結果

結果を以下の表，詳細を添付資料(1)に示す.

濃度	Lot	設定pH	保管温度	保管期間	性状	pH	残存率(%)※2	総類縁物質(%)
1%	Z0204	3	調製直後	−	無色澄明の液	3.05	−	−
			5℃	6d	無色澄明の液	3.12	*100*	1.12
				10d	無色澄明の液	3.08	99.9	0.69
			60℃	6d	無色澄明の液	3.14	97.9	1.13
				10d	無色澄明の液	3.13	98.5	1.78
	Z0205	5	調製直後	−	無色澄明の液，泡立つ	5.01	−	−
			5℃	6d	無色澄明の液，泡立つ	5.02	*100*	0.72
				10d	無色澄明の液，泡立つ	4.98	99.8	0.77
			60℃	6d	無色澄明の液，泡立つ	5.11	99.8	1.19
				10d	無色澄明の液，泡立つ	5.08	99.9	1.26
	Z0206	7	調製直後	−	僅かに白濁した液 泡立つ	6.97	−	−
			5℃	6d	僅かに白濁した液 泡立つ	6.95	*100*	0.71
				10d	僅かに白濁した液 泡立つ	6.90	99.3	0.71
			60℃	6d	僅かに白濁した液 泡立つ	6.99	96.4	3.84
				10d	僅かに白濁した液 泡立つ	6.92	95.6	4.74
0.1%	Z0207	3	調製直後	−	無色澄明の液	3.17	−	−
			5℃	6d	無色澄明の液	3.23	*100*	0.72
				10d	無色澄明の液	3.25	100.8	2.03
			60℃	6d	無色澄明の液	3.23	104.9	1.51
				10d	無色澄明の液	3.21	99.6	1.78
	Z0208	5	調製直後	−	無色澄明の液，僅かに泡立つ	5.07	−	−
			5℃	6d	無色澄明の液，僅かに泡立つ	5.24	*100*	1.17
				10d	無色澄明の液，僅かに泡立つ	5.29	99.7	0.87
			60℃	6d	無色澄明の液，僅かに泡立つ	5.34	104.4	1.50
				10d	無色澄明の液，僅かに泡立つ	5.42	99.5	1.79
	Z0209	7	調製直後	−	無色澄明の液，泡立つ	6.96	−	−
			5℃	6d	無色澄明の液，泡立つ	6.93	*100*	1.21
				10d	無色澄明の液，泡立つ	6.78	99.5	1.11
			60℃	6d	無色澄明の液，泡立つ	7.21	100.3	4.24
				10d	無色澄明の液，泡立つ	7.05	95.1	6.02
0.01%	Z0210	3	調製直後	−	無色澄明の液	3.21	−	−
			5℃	6d	無色澄明の液	3.30	100	0.00
				10d	無色澄明の液	3.29	99.3	0.00 *
			60℃	6d	無色澄明の液	3.33	97.8	0.00 *
				10d	無色澄明の液	3.31	98.9	0.00 *
	Z0211	5	調製直後	−	無色澄明の液，極僅かに泡立つ	5.17	−	−
			5℃	6d	無色澄明の液，極僅かに泡立つ	5.56	100	0.00
				10d	無色澄明の液，極僅かに泡立つ	5.83	98.8	0.00 *
			60℃	6d	無色澄明の液，極僅かに泡立つ	6.67	95.7	0.00 *
				10d	無色澄明の液，極僅かに泡立つ	6.47	*93.4*	1.94 *
	Z0212	7	調製直後	−	無色澄明の液，極僅かに泡立つ	6.85	−	−
			5℃	6d	無色澄明の液，極僅かに泡立つ	6.65	100	0.00 *
				10d	無色澄明の液，極僅かに泡立つ	6.61	100.0	0.00 *
			60℃	6d	無色澄明の液，極僅かに泡立つ	7.28	*94.4*	5.48 *
				10d	無色澄明の液，極僅かに泡立つ	6.99	*92.6*	9.83 *

※2:5℃6日保管品に対する含量%を残存率として算出

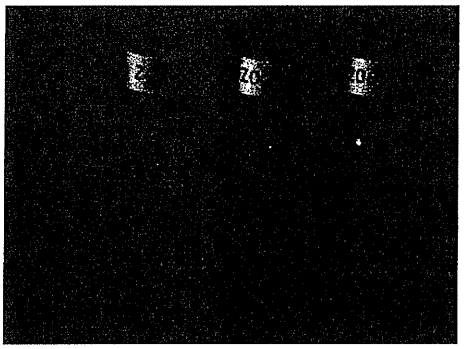
* 製剤の濃度が0.01%（0.1mg/mL）と低濃度であることから，他の濃度と比較して，低濃度の類縁物質は検出できなかったと考えられる

この結果から，下記が確認された．

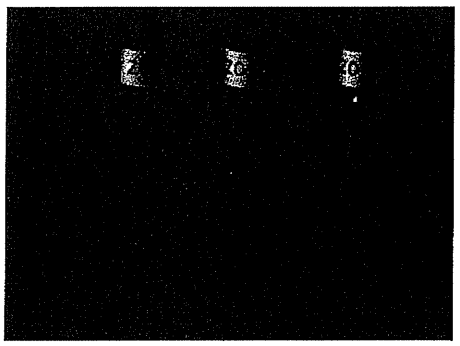
・pHが上昇するに従い，類縁物質が増加する

・いずれの濃度製剤も，pHが上昇するに従い（pH5以上）液性に“泡立ち”が確認された

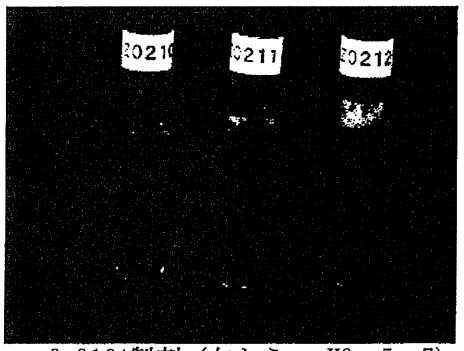
・1%製剤のpH7では，明らかな粘性も認められた．



1%製剤（左から，pH3，5，7），



0.1%製剤（左から，pH3，5，7），



0.01%製剤（左から，pH3，5，7），

図　性状写真（激しく振った後の性状）

これらの結果より，本品のpHは，“pH5.0”以下が望ましいと考えられ，委託者との協議の結果，本品の処方は，pHは4.0に設定した．

6.2. 製造方法及び品質評価

6.2.1. 実施の目的及び概要

本品の濃度0.01%，0.1%，0.3%，1%濃度について試作（0.5Lスケール）を実施し，製造方法を設定する.

6.2.2. 実施内容

1）使用原料

以下の原材料を用いて検証を行った.

原料	製造業者	ロット番号
SR-0379 *	American Peptide	N131003
塩化ナトリウム	ナカライテスク	V3H1038
0.1mol/L 塩酸	ナカライテスク	L3R9080
0.1mol/L水酸化ナトリウム	ナカライテスク	03T9251

*Peptide Content：78.3%

2）処方

以下の処方にて実施した.

原料	1%	0.3%	0.1%	0.01%
	Z0301	Z0304	Z0302	Z0303
SR-0379※	5000mg	1500mg	500mg	50mg
塩化ナトリウム	4.5g	4.5g	4.5g	4.5g
0.1mol/L 塩酸	適量	適量	適量	適量
0.1mol/L 水酸化ナトリウム	適量	適量	適量	適量
精製水	適量	適量	適量	適量
全量	500mL	500mL	500mL	500mL
pH	4.0	4.0	4.0	4.0

※ SR-0379は，Peptide Content(%)により含量換算する.

3）製造方法

① 飽和酢酸カリウム水溶液を用いて，相対湿度30%以下で安定な空間を作り，SR-0379を2時間以上，調湿させる．

② ①のSR-0379を下記の量採取する．

採取必要量＝各理論量 ×100/a

a：peptide content（%）

製剤	1%	0.3%	0.1%	0.01%
	Z0301	Z0304	Z0302	Z0303
SR-0379 理論量	5000mg	1500mg	500mg	50mg

③ 塩化ナトリウム4.5g（4製剤とも）を秤量する．

④ ②③を各々溶解容器に入れ，水を各々450g入れ，撹拌溶解させる．

⑤ 0.1mol/L塩酸又は0.1mol/L水酸化ナトリウムにてpH4.0に調整する．

⑥ 各々全量を500mLとする（比重1.00，質量で合わせる）．

⑦ 本品用スプレー容器に10mLずつ充填し，巻締める．

6.2.3. 実施結果

設定した製法により，液調製が可能であった．またこれらの品質評価結果は以下のとおりであった．微生物限度に関する詳細を添付資料(2)，その他の結果の詳細を添付資料(11)に示す．

品質評価結果

試験項目	規格（案）	0.01%	0.1%	0.3%	1%
		Z0303	Z0302	Z0304	Z0301
性状	無色澄明の液	無色澄明の液	無色澄明の液	無色澄明の液	無色澄明の液
確認試験	保持時間が同様	保持時間が同様	保持時間が同様	保持時間が同様	保持時間が同様
pH	3.0～5.0	4.05	4.03	4.15	4.03
類縁物質	2%以下	0.000	0.845	0.592	0.620
含量	表示量に対し90～110%	91.4	101.9	97.8	100.2
微生物限度	総好気性微生物数 10^2 CFU/mL以下	0 cfu/mL	0 cfu/mL	0 cfu/mL	0 cfu/mL
	総真菌数 10^1 CFU/mL以下	0 cfu/mL	0 cfu/mL	0 cfu/mL	0 cfu/mL
	特性微生物*	検出せず	検出せず	検出せず	検出せず

*黄色ブドウ球菌，緑膿菌

6.3. 容器の選定

容器の選定については8項，容器の安定性評価については10項に詳細を記載した.

6.4. 個装箱の検討

8項に示すとおり，本品の容器は“スプレータイプ樹脂容器”とした.

メーカー	阪本印刷
紙材質	JET スター 310g/m^2

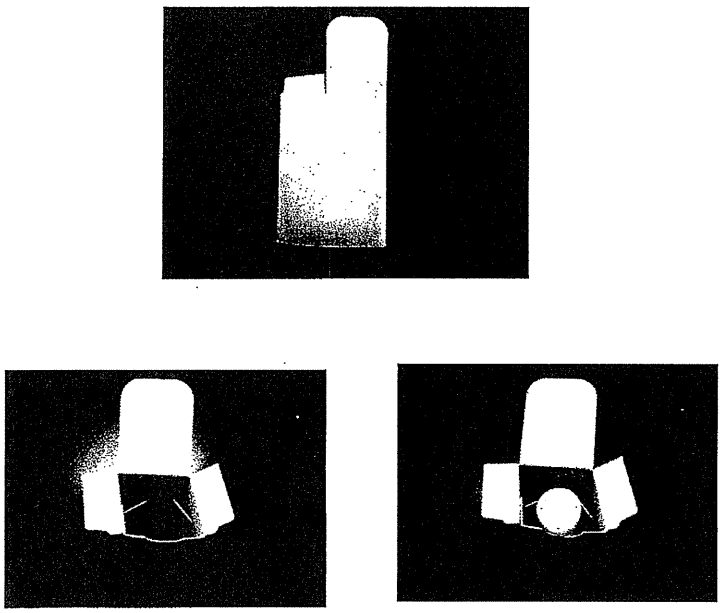


図 本品の個装箱とボトルを入れた状態

7. 市場製品の調査及び容器評価

7.1. 実施の目的及び概要

本品は，難治性皮膚潰瘍患者に一定量を適用する外用液剤となる．そこで，市販の外用液剤を参考にして，本品の容器を設計する．

7.2. 類似する外用液剤の調査

類似する外用液剤として，医療用医薬品，外用液剤の仕様を調査した．

既存の外用液剤（ローション）は多くの製品で用法・用量が“適量をとり，患部に塗布”とされている．本品については，適用量が一定量となる容器が望ましいとの委託先要望により，そのような用法用量の製剤を調査した．

その結果，“フィブラストスプレー250，500”において，用法・用量が規定されていた．(平成26年4月版 保険薬事典)．添付文書及び容器の使用方法説明書を添付資料(3)に示す．

製品例)

商品名	会社名	用法・用量	仕様
ダラシンTローション1%	佐藤製薬	適量を1 日2 回、患部に塗布	20mL PP
アクアチムローション1%	大塚製薬	適量を1 日2 回、患部に塗布	20mLﾌﾟﾗｽﾁｯｸ
フィブラスト スプレー250	科研製薬	例：5噴霧（主薬量として30 μg）	ｽﾌﾟﾚｰﾉｽﾞﾙ付きガラス容器
フィブラスト スプレー500	科研製薬		

7.3. 本品用の容器の選定

フィブラストスプレーは，“1回 噴霧量0.06mL，用法用量は5噴霧，容量は2.5mL又は5mL”である．

容器の選定にあたっては本条件を参考にし，以下の容器を選択した．

仕様		本品の容器	フィブラストスプレー
形状		スプレータイプ	スプレータイプ
容器		白色プラスチック容器	透明ガラス容器
噴霧量／1回		0.05 mL	0.06 mL
噴霧量から換算した使用可能回数		10mL充填として，200回	250製剤(2.5mL)：41回 500製剤(5mL)：83回
メーカー		大成化工株式会社	－
構成	ボトル	PE	－
	スプレー	PP，PE，ｽﾃﾝﾚｽｽﾌﾟﾘﾝｸﾞ	

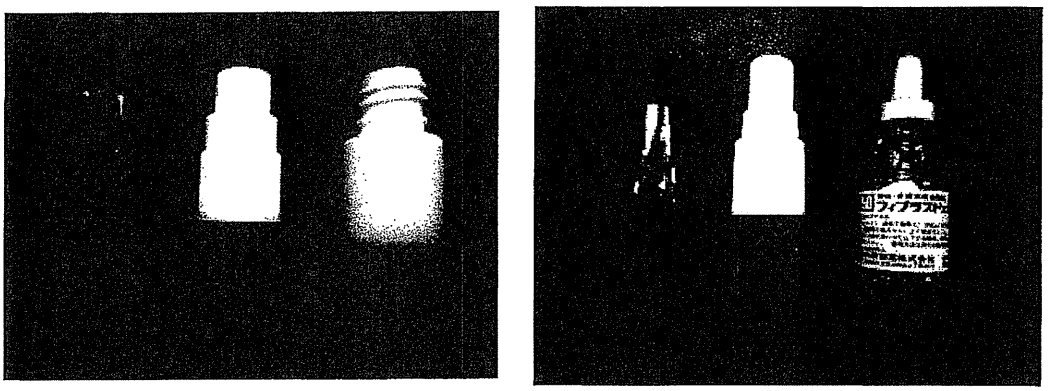


図 本品容器とフィブラストスプレー（左：本品，右：フィブラストスプレー）

7.4. 機能性の調査

①噴霧量

本品及びフィブラストスプレーの噴霧量（精製水）を測定した．結果は下表のとおり，本品の容器（スプレーノズル）は，ほぼ安定した噴霧量を示した．一方，噴霧後の残液量はいずれも3g程度あり，何らかの対策又は充填量の調整が必要と考えられた．結果の詳細を添付資料(4)に示す．

仕様		本品の容器			フィブラストスプレー) *1
		n=1	n=2	n=3	
安定噴霧までの噴霧回数		5	8	7	5
噴霧精度 (g) *2	測定噴霧数	63	56	55	65
	平均噴霧量	0.05406	0.05017	0.05208	0.06307
	σ	0.001	0.007	0.002	0.002
	最小	0.04903	0.00314 0.04748*3	0.04578	0.05759
	最大	0.05556	0.05351	0.05558	0.06904
	幅	0.00653	0.05037	0.00980	0.01145
噴霧後の最終残液量(g)		3.549	3.002	3.018	(実施せず)

*1：スプレーノズルのみ使用して，噴霧量を測定

*2：安定した噴霧回数全データにて算出

*3：0.00314(g)を棄却した場合の最小噴霧量