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European Journal of Pharmacology 390 (2000) 223–228

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Anti-inflammatory action of a novel peptide, SEK-1005, isolated from a *Streptomyces*

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Received 22 November 1999; received in revised form 29 December 1999; accepted 4 January 2000

Abstract

The pharmacological activity of a novel cyclic peptide (SEK-1005: $C_{45}H_{70}N_8O_{13}$) isolated from *Streptomyces nobilis* was studied in rats during the development of inflammation. SEK-1005 (0.1–0.5 mg/kg, i.p.) suppressed the passive Arthus reaction and the carrageenin-induced oedema. A steroidal anti-inflammatory drug, prednisolone (10 mg/kg, i.p.), also was effective on both inflammations. However, indomethacin (5 mg/kg, i.p.), a cyclooxygenase inhibitor, was less effective on the passive Arthus reaction. Also interesting was that the SEK-1005 effect showed its maximum level after a 24-h lag period and that its effect, as well as the prednisolone effect, was reduced by the treatment with a protein synthesis inhibitor, cycloheximide. SEK-1005 and prednisolone also showed marked protection against the adjuvant-induced arthritis, but failed to prevent the tuberculin response. These findings indicate that SEK-1005 is a new type of non-steroidal anti-inflammatory agent with an action similar to that of prednisolone.

Keywords: Inflammation; Steroid-like anti-inflammatory action; Cyclic peptide; *Streptomyce* product

1. Introduction

Steroidal anti-inflammatory medicaments such as prednisolone and dexamethasone are accepted as showing better clinical results than non-steroidal aspirin-like drugs. The results of animal experiments have supported this view, as the steroids are effective on both immunological and non-immunological inflammations unlike the non-steroids, including indomethacin and ibuprofen (Blackham et al., 1975; Chang and Otterness, 1981; Bailey and Sturm, 1983; Reiter et al., 1985; Kuriyama et al., 1988; Hiyama and Kuriyama, 1991; Okamoto et al., 1992). Kuriyama et al. (1988) have confirmed that the steroids are active against every type of swelling response elicited in the rat paw and especially show more potent inhibition on both the passive Arthus reaction and the carrageenin-induced oedema characterized by infiltration with polymorphonuclear leukocytes. From these findings, we have postulated that the passive Arthus reaction would be suitable for seeking out candidate compounds with steroid-like anti-inflammatory activity and fewer side effects. During screening according to this postulate, a novel cyclic peptide (SEK-1005: $C_{45}H_{70}N_8O_{13}$; Fig. 1) (Fujiwara et al., 1999) isolated from *Streptomyces nobilis* was found to be a potent Arthus inhibitor. It was demonstrated here that SEK-1005 markedly alleviates both the passive Arthus reaction and the carrageenin-induced oedema concomitantly with showing interesting properties.

2. Materials and methods

2.1. Animals

The animals were housed for at least 7 days in this laboratory after their arrival. Constant temperature and humidity ($22 \pm 1°C$, $55 \pm 10\%$) were maintained with a fixed 12-h light–dark cycle and free access to food and water. Guiding principles for the care and use of laboratory animals approved by The Japanese Pharmacological Society were followed in this animal study.

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00017-0

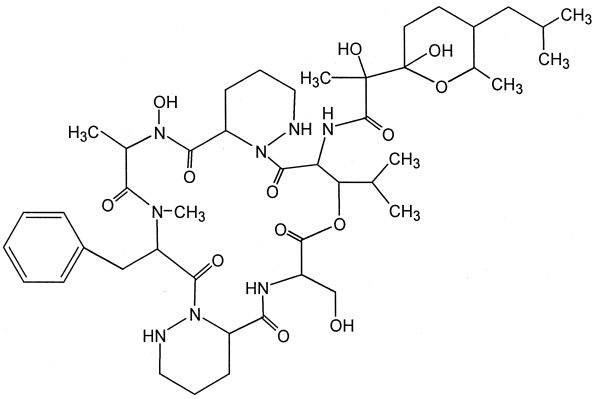

Fig. 1. Chemical structure of SEK-1005: Ser, 3-hydroxy-*N*-[2-hydroxy-1-oxo-2-tetrahydro-2-hydroxy-6-methyl-5-(2-methylpropyl)-2 *H*-pyran-2-yl-propyl]-Leu-Pip(hexahydro-3-pyridazinecarbonyl)-*N*-hydroxy-Ala-*N*-methyl-Phe-Pip-rho-lactone.

2.2. Compounds

SEK-1005 [purity: more than 96% by UV detection at 210 nm by reverse phase high-performance liquid chromatography; content of endotoxins: less than 1 EU/g by a commercial limulus assay (Endospecy) (Seikagaku, Tokyo, Japan)] was prepared in this laboratory as described previously (Fujiwara et al., 1999). Prednisolone (Wako, Osaka, Japan), indomethacin (Sigma, St. Louis, USA) or SEK-1005 was suspended in 5% gum arabic (Nacarai tesque, Kyoto, Japan) just prior to use and injected intraperitoneally or subcutaneously in a volume of 2 ml/kg for all experiments. SEK-1005 was not available for intravenous injection, being almost insoluble in water. Cycloheximide (Wako) was dissolved in saline to be injected intraperitoneally.

2.3. Passive skin Arthus reaction

Rabbit antiserum against ovalbumin (Sigma) was prepared as follows. New Zealand albino male rabbits (Keari, Osaka, Japan) weighing about 3 kg were immunized with an equivolume mixture of ovalbumin (20 mg/ml) in saline and Freund's complete adjuvant (Difco Laboratories, Detroit, USA). One milliliter of the mixture on day 0 (first immunization) and 2 ml each on days 7, 14 and 21 were injected intramuscularly into both thighs. The antiserum was collected on day 28. The titer of antiserum was 1:8000, as estimated by passive cutaneous anaphylaxis test with 4-h latent period in Hartley male guinea pigs (Japan SLC, Hamamatsu, Japan) weighing 350–400 g. The antiserum diluted 1:4 with saline was injected intradermally 0.05 ml/site onto the shaved back of male Wistar rats (Japan SLC) weighing 160–200 g. Four hours later, 0.5% Evan's blue saline containing ovalbumin 2 mg/ml was injected intravenously 0.5 ml/kg to induce the passive skin Arthus reaction. The animals were killed 1 h after antigen challenge. The skin of the Arthus lesion was taken to measure the amount of leaked dye according to the method of Harada et al. (1971).

2.4. Carrageenin-induced paw oedema

One-tenth milliliter of 1% λ-carrageenin (Picnin A, Zushi Chemicals, Zushi, Japan) dissolved in saline was injected subcutaneously into a footpad of Wistar male rats weighing 150–190 g. Paw volume was measured just before and 4 h after carrageenin injection with a plethysmometer (Ugo Basile, Milano, Italy). Swelling volume was determined by the difference from the initial paw volume.

2.5. Tuberculin skin response

The skin response was induced according to the method of Hiyama et al. (1989). Male Wistar rats weighing 180–220 g were intraperitoneally injected with heat-inactivated (121°C, 5 min) Bacillus Calmette–Guerin (Nippon BCG, Tokyo, Japan) suspended in saline (0.5 mg/0.2 ml/rat). After 7 days, 0.1 ml of purified tuberculin (PPD, Nippon BCG) in saline (20 mg/ml) was injected intradermally to induce the delayed-type skin response. Twenty-four hours later, the diameter of the erythema was measured to evaluate the effects of compounds to be tested.

2.6. Adjuvant-induced arthritis

Male Lewis rats (Charles River Japan, Yokohama, Japan) weighing 190–200 g were used. Primary adjuvant arthritis was induced in the right hind paw by the subplantar injection (0.1 ml) of liquid paraffin containing *Mycobacterium tuberculosis* H37RA (Difco Laboratories) 6 mg/ml. Paw volumes were measured daily to determine the swelling volume as described for carrageenin-induced oedema.

2.7. Statistical analysis

The data are presented as the means ± S.E.M. Statistical analysis involving two groups was performed with Student's *t*-test, and Dunnett's multiple comparison test was used for more than two groups. P values < 0.05 were considered to be significant.

3. Results

3.1. Effect on the passive skin Arthus reaction

SEK-1005 was given intraperitoneally at appropriate time intervals before elicitation of the Arthus reaction. A dye leakage of 11.5–15.6 μg/site was induced at the Arthus lesion in control animals. SEK-1005 (0.5 mg/kg, i.p.) produced a time course change with maximum sup-

Table 1
Time course change of anti-inflammatory effect of SEK-1005 on passive skin Arthus reaction in rats
Each figure indicates the mean ± S.E.M. for three animals.

Time interval (h)	Amount of dye (μg/site)		Inhibition (%)
	Control	SEK-1005 (0.5 mg/kg)	
1	12.8 ± 1.0	7.9 ± 0.1[a]	38.3
3	12.2 ± 0.7	2.8 ± 0.1[a]	77.0
6	14.8 ± 0.6	2.1 ± 0.4[a]	85.8
24	14.2 ± 1.4	0.5 ± 0.4[a]	96.5
48	13.0 ± 1.6	2.7 ± 1.3[a]	79.2
72	13.5 ± 1.3	8.4 ± 2.0	37.8

[a] $P < 0.01$: significant relative to the control.

pression of this response after a 24-h lag period (Table 1). The inhibition (%) was more than 70% with doses between 3 and 48 h before antigen challenge. The priming with SEK-1005 (i.p.) 24 h prior to the inflammatory insult produced a dose-dependent inhibition significantly different from the control at the doses of 0.5 and 0.1 mg/kg as shown in Table 2. SEK-1005 also significantly suppressed the Arthus reaction at subcutaneous doses of 0.2 and 1 mg/kg (−24 h). However, the compound (0.5–5 mg/kg) was inactive when given orally 24 h previously. Reference compounds, prednisolone and indomethacin, were administered intraperitoneally 1 h before elicitation to produce their near-maximum effects. The steroidal anti-inflammatory agent, prednisolone (10 mg/kg), was active against the Arthus reaction with inhibition of about 50%, although it was less potent than SEK-1005 (0.5 mg/kg). Indomethacin, a non-steroidal agent, showed only an inhibitory tendency at a dose of 5 mg/kg.

3.2. Effect on the carrageenin-induced oedema

SEK-1005 or reference compounds were administered intraperitoneally 24 or 1 h prior to carrageenin injection, respectively. Paw swelling (0.49–0.67 ml) developed in the control over 4 h after elicitation. SEK-1005 (0.1 and 0.5 mg/kg) markedly reduced the paw swelling (Table 3). The effect of SEK-1005 even at 0.1 mg/kg was more potent than that of prednisolone (10 mg/kg) and almost comparable to the activity of indomethacin (5 mg/kg).

Table 2
Effects of SEK-1005, prednisolone and indomethacin on passive skin Arthus reaction in rats
Each figure indicates the mean ± S.E.M. for four to five animals.

Compound	Dose (mg/kg)	Amount of dye (μg/site)	Inhibition (%)
Control (i.p.)	–	15.3 ± 1.5	
SEK-1005 (i.p.)	0.5	0.8 ± 0.2[a]	94.8
	0.1	9.6 ± 0.8[b]	37.3
	0.02	11.1 ± 1.4	27.5
Control (s.c.)	–	10.3 ± 1.4	
SEK-1005 (s.c.)	1	3.1 ± 1.7[b]	69.9
	0.2	5.0 ± 0.9[b]	51.5
	0.05	7.5 ± 1.1	27.2
Control (i.p.)		13.5 ± 1.7	
Prednisolone (i.p.)	10	7.1 ± 1.1[b]	47.4
Indomethacin (i.p.)	5	12.4 ± 1.4	8.1

[a] $P < 0.001$: significant relative to the control.
[b] $P < 0.05$: significant relative to the control.

Table 3
Effects of SEK-1005, prednisolone and indomethacin on carrageenin-induced paw oedema in rats
Each figure indicates the mean ± S.E.M. for three to five animals.

Compound	Dose (mg/kg)	Swelling volume (ml)
Control	–	0.49 ± 0.03
SEK-1005	0.5	0.01 ± 0.02[a]
	0.1	0.18 ± 0.03[b]
Control		0.67 ± 0.07
Prednisolone	10	0.48 ± 0.09
Indomethacin	5	0.26 ± 0.06[c]

[a] $P < 0.001$: significant relative to the control.
[b] $P < 0.01$: significant relative to the control.
[c] $P < 0.05$: significant relative to the control.

3.3. Effect on the tuberculin skin response

SEK-1005 or prednisolone was administered intraperitoneally 1 h before or 6 h after PPD injection, respectively. These dosing times were determined from the results in Table 1 and the report of Hiyama et al.(1989). As apparent from Table 4, SEK-1005 at 0.1 and 0.5 mg/kg had almost no effect on erythema diameter. Prednisolone at 10 mg/kg had only a slight anti-inflammatory activity.

3.4. Effect on the adjuvant-induced arthritis

SEK-1005 (i.p.) or prednisolone (i.p.) was administered repeatedly, once a day for 22 days, from day 0. Primary arthritis (right hind paw) developed with time following the adjuvant injection and reached its maximal level on days 14–16. The secondary arthritis (left hind paw), immunologically caused, appeared on days 5–10 and thereafter showed a time course change similar to that of the primary response.

The severity of the arthritis was clearly reduced in both groups of animals receiving SEK-1005 and prednisolone. SEK-1005 suppressed both primary and secondary arthritis in a dose-dependent manner, showing a significant differ-

Table 4
Effects of SEK-1005 and prednisolone on tuberculin skin response in rats
Each figure indicates the mean ± S.E.M. for four to five animals.

Compound	Dose (mg/kg)	Erythema diameter (mm)
Control	–	10.3 ± 1.0
SEK-1005	0.5	9.2 ± 0.7
	0.1	9.0 ± 1.2
Control		10.5 ± 0.8
Prednisolone	10	9.0 ± 0.9

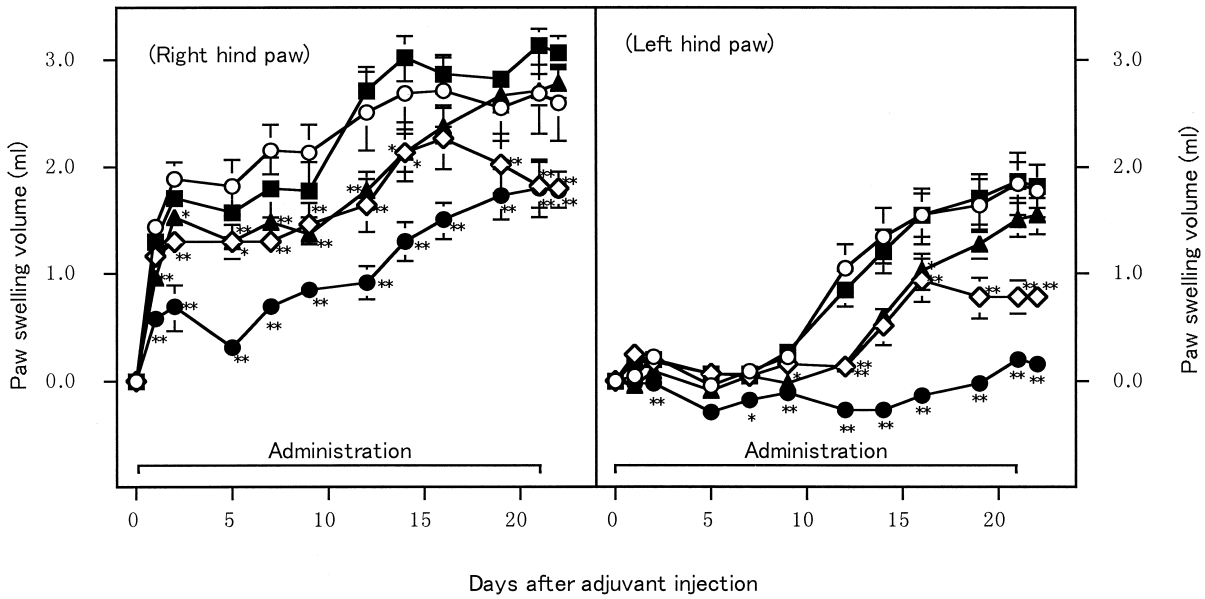

Fig. 2. Effects of SEK-1005 and prednisolone on adjuvant-induced arthritis in rats. Each point and vertical bar indicates the mean ± S.E.M. for seven animals. $^{*}P < 0.05$, $^{**}P < 0.01$: significant relative to the control. (○) Control. (■) SEK-1005, 0.02 mg/kg. (▲) SEK-1005, 0.1 mg/kg. (●) SEK-1005, 0.5 mg/kg. (◇) Prednisolone, 10 mg/kg.

ence from the control at doses of 0.1 and 0.5 mg/kg, but there was no effect at a dose of 0.02 mg/kg. Prednisolone at 10 mg/kg also exhibited significant anti-inflammatory activity, although it was less effective than SEK-1005 at 0.5 mg/kg (Fig. 2).

All animals were autopsied on day 22. There was no macroscopic evidence of adverse effects in the group receiving SEK-1005 while atrophy of thymus and adrenal gland was produced in all seven animals treated with prednisolone.

3.5. Effect of cycloheximide on anti-inflammatory activity of SEK-1005

Compounds to be tested were administered 3 h before initiation of inflammation. The animals were given cycloheximide (1 mg/kg, i.p.) twice, 90 and 120 min, before elicitation. SEK-1005 (0.5 mg/kg, i.p.) and prednisolone (10 mg/kg, i.p.) had a similar anti-inflammatory property (Table 5). Both compounds were significantly active on the histamine-induced response in addition to the Arthus reaction. Furthermore, these anti-inflammatory actions were apparently reduced by the administration of an inhibitor of protein biosynthesis, cycloheximide. This suggests that SEK-1005 induces the synthesis of protein(s) responsible for its anti-inflammatory actions.

4. Discussion

The results presented here have demonstrated that a novel peptide, SEK-1005, is a new type of anti-inflammatory agent. To our knowledge, this is the first demonstration that the product derived from *S. nobilis* has an anti-inflammatory action.

Table 5
Effect of cycloheximide on anti-inflammatory acitons of SEK-1005 and prednisolone
Each figure indicates the mean ± S.E.M. for four (passive Arthus) or five (histamine response) animals.

Compound	Cycloheximide (i.p.)	Amount of leaked dye (μg/site)	
		Passive Arthus	Histamine response
Control	−	14.0 ± 1.4	16.3 ± 2.0
	+	7.7 ± 1.0	11.3 ± 1.5
SEK-1005 (0.5 mg/kg, i.p.)	−	1.8 ± 0.6[a]	4.6 ± 1.1[b]
	+	6.1 ± 1.6	13.7 ± 1.5
Prednisolone (10 mg/kg, i.p.)	−	3.2 ± 0.6[b]	8.7 ± 0.3[b]
	+	6.2 ± 0.5	12.2 ± 2.5

[a] $P < 0.01$: significant relative to the control.
[b] $P < 0.05$: significant relative to the control.

Since penicillin separated from fungi products was found to be a potent antibiotic, the search for natural products available for therapy has remained active. At present, in addition to many antibiotics, immunosuppressors (Borel et al., 1977; Britton and Palacios, 1982; Kino et al., 1987a,b) and 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor for treating hyperlipidemia (Tsujita, 1993) have been practically applied as useful medicaments derived from natural sources. Our aim was to find products derived from *Streptomyces* in a search for a new type of anti-inflammatory agent. In the screening test for about hundred strains, some candidate strains were demonstrated to produce anti-inflammatory factor(s) effective against the skin passive Arthus reaction. One of these was *S. nobilis*, producing a novel peptide, SEK-1005.

SEK-1005 had an interesting pharmacological activity as it was effective on both immunological Arthus reaction and non-immunological carrageenin-induced oedema. Considering that immunological inflammations such as the Arthus reaction could initiate and/or promote chronic inflammatory disorders (Fernandez et al., 1990; Fujii et al., 1990; Nabozny and David, 1994; Nahm and Park, 1996; Zeuner et al., 1996; Madaio, 1999) including rheumatoid arthritis and that the suppression of the immunological inflammations by anti-inflammatory steroids plays an important role in their clinical effects, it is especially significant that SEK-1005 as well as prednisolone showed potent suppression of the Arthus reaction, on which a non-steroidal anti-inflammatory drug, indomethacin, was little effective. It is also interesting that SEK-1005 inhibits the Arthus reaction with a time lag. The detailed mechanism of this delayed-type effect is still unclear. But SEK-1005 is postulated to exert its activity indirectly through increasing the synthesis of endogenous anti-inflammatory protein(s), as reported for anti-inflammatory steroids (Tsurufuji et al., 1979; Barnes, 1996; Ahluwalia, 1998; Almawi et al., 1998). Indeed, the anti-inflammatory activity of SEK-1005, as well as of prednisolone, was depressed by the treatment with cycloheximide, an inhibitor of protein synthesis.

Furthermore, based on the following data, SEK-1005 appears to increase the synthesis of anti-inflammatory protein(s) as do vessel endothelial stabilizer(s) reported for the steroids (Oyanagi and Suzuki, 1985; Carnuccio et al., 1987). Both SEK-1005 and prednisolone exhibited a broad spectrum of effects against inflammations, including the histamine response. Histamine stimulates vessel endothelial cells to increase vascular permeability. SEK-1005 markedly inhibited the histamine-induced increase in permeability and this activity was reduced by the treatment with cycloheximide. The same was true for prednisolone. These results suggest that SEK-1005 and prednisolone might have common pharmacological activity on vessel endothelium. In addition, both compounds were ineffective against the skin erythema induced by tuberculin hyperreactivity, suggesting that they might possess a similar specificity of action on vessel endothelium.

Delayed-type anti-inflammatory activity has been also demonstrated with tilorone, a potent interferon inducer. Tilorone has been reported to be effective on both inflammations, the passive Arthus reaction and the carrageenin-induced oedema with a specific time lag (Megel et al., 1975; Hiyama and Kuriyama, 1991). Although the anti-inflammatory action of tilorone is suggested to be dissociated from its interferon-inducing activity (Hiyama and Kuriyama 1991), it is not excluded that SEK-1005 might have an anti-inflammatory activity related to interferon induction because anti-inflammatory activity, such as the suppression of adjuvant-induced arthritis, was demonstrated with another interferon-inducing agent (Kapusta and Mendelson, 1969).

Further studies regarding anti-inflammatory protein(s) responsible for the SEK-1005 effect are now in progress. A preliminary experiment suggested that transforming growth factor (TGF)-β_1 might possibly be one of the protein(s) responsible. Thus, SEK-1005 applied topically is shown to accelerate the production of TGF-β_1 in a wound healing model with mice. The cytokine has been demonstrated to be an endogenous inhibitor of inflammation (Ulich et al., 1991; Meade et al., 1992; Shull et al., 1992), and anti-inflammatory steroids have been recently described (Almawi et al., 1998) to induce TGF-β_1 expression. These findings support the evidence given here that SEK-1005 has an anti-inflammatory action similar to that of prednisolone.

The effect of SEK-1005 against chronic inflammation was evaluated in rats with adjuvant-induced arthritis. The compound provided remarkable protection against the swelling development of arthritis, without the atrophy of thymus and adrenal gland observed in animals receiving prednisolone. This result indicates that SEK-1005 might be an arthritis inhibitor with lower toxicity than prednisolone. However, a sensitive-enough test, including the measurement of corticosterone blood levels, would be required to define better if the endogenous steroid contributes to the SEK-1005 effect or not.

From the above, it appears that SEK-1005 is a new type of non-steroidal anti-inflammatory peptide, with a steroid-like action, also throwing new light on the search for still better anti-inflammatory medicaments.

Acknowledgements

We are grateful to Mr. T. Shimizu for technical assistance.

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