Inhibition of fibroblast-like cell proliferation by interleukin-1 blockers, CK-119 and CK-122

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AIM: To study potent and nontoxic agents to inhibit fibroblast proliferation. **METHODS**: Fibroblast-like corneal and conjunctival cells were cultured and inhibited by interleukin-1 (IL-1) blockers, dihydropyridazino-pyridazines CK-119 and CK-122. The cell growth and syntheses of DNA, RNA, and protein after IL-1 blocker incubation were determined. **RESULTS**: CK-119 and CK-122 inhibited cell growth of corneal fibroblast at 30 mg $\cdot$ L^{-1} or higher whereas conjunctival cells were inhibited at a concentration as low as 3 mg $\cdot$ L^{-1}. DNA and RNA syntheses in corneal fibroblasts were markedly inhibited by CK-119 and CK-122 whereas protein synthesis was either unaffected or mostly enhanced at 30 – 100 mg $\cdot$ L^{-1} and 100 – 300 mg $\cdot$ L^{-1}, respectively. Similar results were obtained in conjunctival cell cultures by CK-119 and CK-122 at 3 – 10 mg $\cdot$ L^{-1} and 30 – 100 mg $\cdot$ L^{-1}, respectively. **CONCLUSION**: CK-119 and CK-122 are potent IL-1 blockers to inhibit cell growth of fibroblast-like corneal and conjunctival cells mainly through the inhibition of DNA and RNA syntheses but not protein synthesis.

The effectiveness of interleukin-1 receptor antagonist (IL-1ra) for treatment of numerous inflammatory diseases[1-5] triggered a search of a new class of non-steroidal anti-inflammatory drugs (NSAID) other than aracidonate metabolite inhibitors[6-8]. Since IL-1ra is an effective but unstable peptide with $T_{1/2}$ of only 21 min, stable chemicals with a longer duration of action are

sought[9-13]. Dihydropyridazino-pyridazine derivatives CK-119 and CK-122 are potent IL-1 blockers to inhibit posterior uveitis induced by intravitreously injected IL-1[13]. IL-1 blockers were extremely safe to be used systemically with LD$_{50}$ > 25 g $\cdot$ kg^{-1} *po* and therapeutic index (LD$_{50}$/ED$_{50}$) $\geqslant$ 1000. Numerous drugs such as mytomycin C, 5-fluorouracil, etc, have been used in the clinics to prolong the functional period of aqueous humor drainage cannula created in the filtration surgery (trabeculectomy)[14,15]. Unfortunately, all these agents are quite toxic to induce serious side effects. Since IL-1 blockers showed a very high therapeutic index, they should be able to replace these agents to produce better efficacy and safety.

Surgical injury results in inflammation and fibroblast proliferation which are stimulated by IL-1, therefore, IL-1 blockers are useful in prolonging the functional period of passage cannula created by filtration surgery (trabeculectomy) in low-tension glaucoma or close-angle glaucomas[14,15].
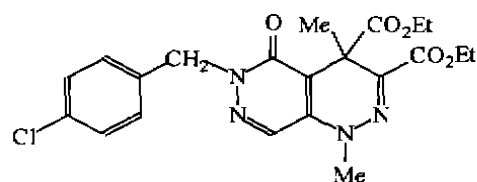
CK-119, C$_{21}$H$_{23}$ClN$_4$O$_5$ (446.89)
mp 149 – 150 °C, soluble in MeOH, EtOH, CH$_2$Cl$_2$
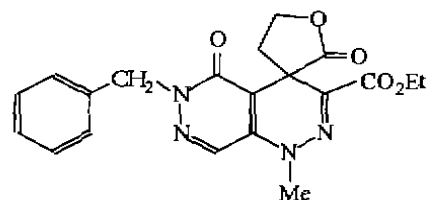
CK-122, C$_{20}$H$_{20}$N$_4$O$_5$ (382.38)
mp 159 – 160 °C, soluble in MeOH, EtOH, CH$_2$Cl$_2$

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To understand the effect of IL-blockers on fibroblast proliferation, a study of CK-119 and CK-122 on fibroblast-like corneal and conjunctival cells in the cell culture were carried out. Further, to elucidate the mechanism of cell growth inhibition, effects of these compounds on the syntheses of protein, mRNA, and DNA were also investigated.

MATERIALS AND METHODS

Materials CK-119 and CK-122 were synthesized[11 - 13]. Hyamine hydroxide, (a cell solubilizer), CytoScint scentillation cocktail, [^{3}H]leucine 4477 TBq · mol^{-1}, [^{3}H]uridine 1628 TBq·mol^{-1} and [^{3}H]thymidine 2368 TBq ·mol^{-1} were purchased from ICN Radiochemicals (Irvine CA). Eagle's minimum essential medium (MEM), Medium 199, and antibiotic-antimycotic (penicillin G 10 MU·L^{-1}, streptomycin sulfate 10 g·L^{-1}, and amphotericin B 25 mg · L^{-1}) were obtained from Grand Island Biological Co (Grand Island NT). Me$_2$SO and fetal bovine serum were purchased from Sigma Chemical Co (St Louis MO). All solutions of the CK-compounds were prepared in pure Me$_2$SO and then diluted in the culture medium. Equal amounts of Me$_2$SO were used as a control vehicle. The final concentration of Me$_2$SO in the cell culture was 1 %.

Cell cultures SIRC cells, a stable fibroblast cell line derived from rabbit cornea, were obtained from American Type Culture Collection (Rockville MD). Cells were grown in monolayer in 75-cm^2 flasks in MEM with glutamine supplementation plus 10 % fetal bovine serum, benzylpenicillin 100 kU · L^{-1}, streptomycin 100 mg·L^{-1}, and amphotericin B 0.25 mg·L^{-1}. The cultures were kept at 37 ℃ in a 100 % humidified chamber of 5 % CO$_2$ + 95 % air, and the medium was changed every 2 - 3 d. Cells reached confluence in 6 d and were replaced by trituration with 0.2 % trypsin plus edetic acid in MEM.

Clone 1 - 5c - 4 Wong-Kilbourne derivative (D) human fibroblast-like conjunctival cells were purchased from American Type Culture Collection (Rockville MD). Cells were grown in monolayer in 75-cm^2 flasks in M199 with Hanks' salts and L-glutamine supplementation plus 10 %

fetal bovine serum, benzylpenicillin 100 kU ·L^{-1}, streptomycin 100 mg ·L^{-1}, and amphotericin B 0.25 mg · L^{-1}. The cultures were incubated at 37 ℃ in a 100 % humidified chamber in the air atmosphere, and the medium was changed every 2 - 3 d. Cells reached confluence in 6 d and were replaced by trituration with 0.25 % trypsin plus edetic acid in Hanks' balanced salt solution (HBSS).

Measurement of cell proliferation For cytotoxicity studies, cells were harvested and diluted with MEM to 2 × 10^8 corneal cells · L^{-1} and M199 to 3 × 10^8 conjunctival cells · L^{-1}. They were seeded (3 mL) in 6-well culture dishes for 24 h for corneal cells and 48 h for conjunctival cells. The CK-compounds or the vehicle were then added to the dishes. Cells were allowed to grow with the CK-compound-containing medium for 24, 48, and 72 h for corneal cells or 48, 72, 96, and 120 h for conjunctival cells. The viable cells were counted at each time point. The cells were triturated with 0.2 % trypsin plus edetic acid and then counted using a hemocytometer. Cell viability was determined by the exclusion method with 0.4 % trypan blue.

Measurement of ^{3}H incorporation For viability studies, cells were cultured in 6-well dishes for 24 h for corneal cells and 48 h for conjunctival cells in the absence of CK-compounds. The CK-pounds and vehicle were then added to the dishes. At the end of 24, 48, and 72 h for fibroblast-like corneal cells or 48, 72, 96, and 120 h for conjunctival cells, 6 dishes were triturated for cell counts using a hemocytometer, and cell viability was determined with the trypan blue exclusion method. Other dishes were given [^{3}H]leucine (18.5 MBq ·L^{-1}, 4477 TBq·mol^{-1}), [^{3}H]uridine (1.85 MBq ·L^{-1}, 1628 TBq·mol^{-1}) or [^{3}H]thymidine (18.5 MBq ·L^{-1}, 2368 TBq · mol^{-1}). Cells were incubated with the isotopes for 6 h. The cell medium was decanted; the cells adhering to the dish were washed with cold phosphate-buffered saline (PBS) thrice and were solubilized with 0.8 mL of hyamine hydroxide solubilizer to rlease the incorporated radioactivity. A 0.2-mL aliquot of dissolved cells was transferred to 5 mL CytoScint counting solution, and the radioactivity was counted with a

Beckman LS 5000 CE Beta counter.

Data analysis   All data were presented as $\bar{x} \pm s$. The number of viable cells was determined and the syntheses of DNA, RNA, or protein were expressed as $Bq/10^5$ cells per 6 h. Statistical analyses utilized t-test and two-way ANOVA.

RESULTS

When fibroblast-like corneal cells were incubated with CK-119, the cultured cell growth was inhibited with concentration-response relationship as 30, 100, and 300 mg·L^{-1}(Fig 1). CK-119 was not a cytolytic compound as the cells were growing at a slower rate but were not dead. CK-122 showed similar inhibition results though much less potent than CK-119 at the same concentrations used (Fig 1).
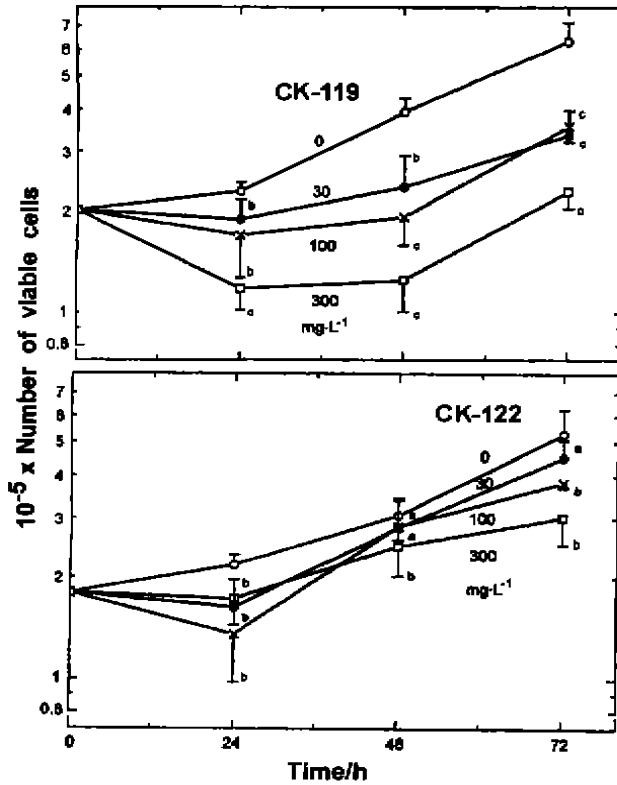

Fig 1.   Effects of CK-119 and CK-122 on fibroblastic cornea cells. n = 6 wells, $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ vs corresponding controls.

When conjunctival cell cultures were incubated with CK-119, marked inhibition of cell growth was seen at concentrations 1/10 those of CK-122 (Fig 2).   Conjunctival cells were
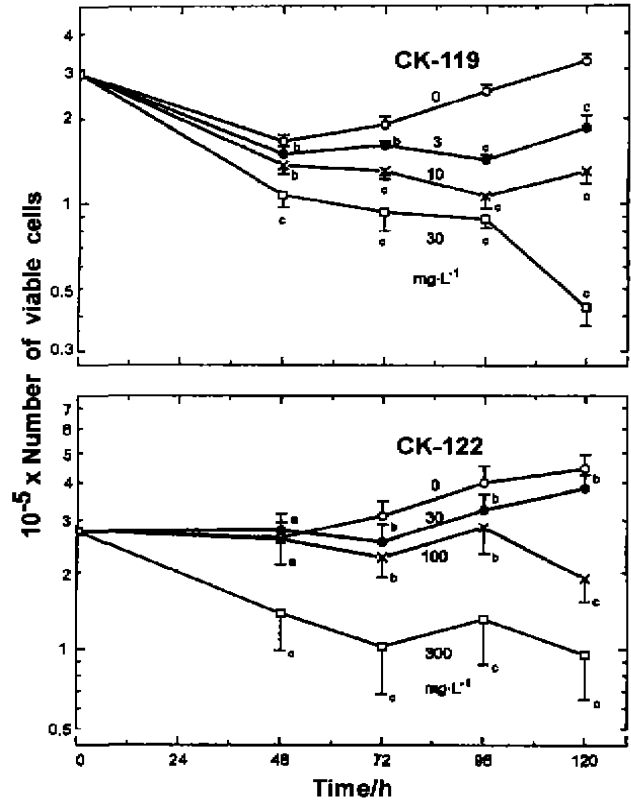

Fig 2.   Effects of CK-119 and CK-122 on conjunctival cells. n = 6 wells, $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ vs corresponding controls.

inhibited effectively by CK-119 3, 10, and 30 mg·L^{-1} whereas corneal cells required CK-119 30, 100, and 300 mg·L^{-1} to achieve the same degree of inhibition (Fig 1, 2).

When CK-119 was incubated with fibroblast-like corneal cells, it inhibited thymidine (DNA synthesis) and uridine (RNA synthesis) incorporation markedly at 30 and 100 mg·L^{-1}. DNA synthesis was inhibited 74 % – 90 % at 30 and 47 % – 83 % at 100 mg·L^{-1}. In case of RNA synthesis, it was inhibited 60 % – 89 % at 30 mg·L^{-1} and 40 % – 80 % at 100 mg·L^{-1}. On the other hand, protein synthesis was unaffected or slightly increased by 15 % at both 30 and 100 mg · L^{-1}. These results indicated that cell growth inhibition caused by CK-119 was mainly due to inhibition of DNA and RNA and was unrelated to protein synthesis (Tab 1).

Similar results were obtained when fibroblast-like corneal cells were incubated with CK-122.   At 100 mg · L^{-1}, DNA and RNA syntheses were inhibited 64 % – 80 % and 74 % – 78 %, respectively.   At 300 mg·L^{-1},

DNA and RNA syntheses were inhibited 73 % – 85 % and 82 % – 93 %, respectively. On the other hand, protein syntheses were enhanced up to 45 %, indicating that, again, cell growth inhibition by CK-122 was caused mainly by the inhibition of DNA and RNA synthesis but not related to inhibition of protein synthesis (Tab 1).

Fibroblast-like conjunctival cells were much more sensitive (approximately 10-fold difference) to CK compounds inhibition than corneal cells (Tab 2). At 3 mg·L^{-1} of CK-119, DNA and RNA syntheses were inhibited 9 % – 61 % and 40 % – 48 %, respectively, after 72 – 120-h incubation. At 10 mg·L^{-1} of CK-119, DNA and RNA syntheses were inhibited 71 % – 77 % and 73 % – 81 %, respectively after 72 – 120-h incubation. Protein synthesis was only slightly inhibited (8 % – 14 %) by CK-119 after 120-h incubation but in all other time points, protein syntheses were either unaffected or markedly enhanced by CK-119 up to 48 % (Tab 2).

Similar results were obtained with CK-122 on fibroblast-like conjunctival cell inhibition. At 30 mg·L^{-1}, DNA and RNA syntheses were inhibited 34 % – 64 % and 60 % – 76 %, respectively. At 100 mg·L^{-1}, DNA and RNA syntheses were inhibited 45 % – 92 % and 87 % – 95 %, respectively (Tab 2). Protein synthesis was unaffected at 48 h and 72 h but was increased 21 % – 30 % at 96 h and 120 h at 30 mg·L^{-1}. At 100 mg·L^{-1}, the protein synthesis was increased 24 % at 48 h, suppressed 19 % – 35 % at 72 – 96 h, and unaffected at 120 h after incubation (Tab 2). These results indicated that inhibition of conjunctival cell growth was similar to that of corneal cell inhibition via DNA and RNA syntheses but not protein synthesis.

Tab 1. Effects of CK-119 and CK-122 on incorporation of thymidine, uridine, and leucine into fibroblast-like corneal cells. A: 33±9 Bq/10^5 cells, B: 84±7 Bq/10^5 cells, C: 22.2±2.3 Bq/10^5 cells; D: 9.9±1.8 Bq/10^5 cells, E: 78±3 Bq/10^5 cells, F: 14.8±0.9 Bq/10^5 cells. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs control.

| Drug/ mg·L^{-1} | ^{3}H incorporation/% ($n = 6$ wells, $\bar{x} \pm s$) | | | | | | | | |
| | Thymidine | | | Uridine | | | Leucine | | |
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
CK-119									
0	100^A	100^A	100^A	100^B	100^B	100^B	100^C	100^C	100^C
30	9.5±2.0^c	26±3^c	25.4±2.5^c	10.9±1.0^c	18.4±1.3^c	38±3^c	101±8^a	115±6^b	98±6^a
100	17±3^c	46±12^c	53±5^c	20±3^c	41±3^c	60±4^c	109±12^a	115±5^b	99±9^a
CK-122									
0	100^D	100^D	100^D	100^E	100^E	100^E	100^F	100^F	100^F
100	36±7^c	34±3^c	19.6±2.3^c	21.6±1.3^c	26.4±1.3^c	25.9±2.0^c	139±6^b	103±5^a	91±4^b
300	14.6±2.0^c	17±4^c	26±5^c	6.8±0.3^c	7.8±1.0^c	17.8±1.3^c	144±10^c	113±7^b	140±9^c

Tab 2. Effects of CK-119 and CK-122 on incorporation of thymidine, uridine, and leucine into conjunctival cells. A: 4.0±0.6 Bq/10^5 cells, B: 41±4 Bq/10^5 cells, C: 11.0±0.7 Bq/10^5 cells; D: 3.2±0.5 Bq/10^5 cells, E: 41.9±2.8 Bq/10^5 cells, F: 10.7±1.1 Bq/10^5 cells. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs control.

| Drug/ mg·L^{-1} | ^{3}H incorporation/% ($n = 6$ wells, $\bar{x} \pm s$) | | | | | | | | | | | |
| | Thymidine | | | | Uridine | | | | Leucine | | | |
	48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h
CK-119												
0	100^A	100^A	100^A	100^A	100^B	100^B	100^B	100^B	100^C	100^C	100^C	100^C
3	100±12^a	91±12^a	67±11^c	39±4^c	90±7^a	59±5^c	60±4^c	52±5^c	148±8^c	107±10^a	123±6^b	91.5±2.5^b
10	32±6^c	29.3±2.0^c	27±7^c	23±6^c	22.5±2.3^c	27±4^c	19.2±1.5^c	21.6±2.3^c	116±4^b	96±6^a	106±6^a	86±6^c
CK-122												
0	100^D	100^D	100^D	100^D	100^E	100^E	100^E	100^E	100^F	100^F	100^F	100^F
30	54±7^c	36±3^c	66±18^c	46±7^c	23.8±2.5^c	34.0±2.5^c	38.0±2.0^c	40±5^c	103±8^a	106±6^a	130±13^c	121±7^c
100	55±10^c	8.5±1.5^c	8.1±1.3^c	22.2±2.8^c	5.2±0.8^c	6.0±0.5^c	5.8±0.5^c	12.9±1.5^c	124±8^c	65±4^c	81±6^c	112±7^b

DISCUSSION

There are a large number of narrow-angle or close-angle glaucoma patients who need filtration surgery to drain the excess aqueous humor. The major reason of failure in filtration surgery is the blockade of drainage cannula by fibroblast proliferation and scar formation. There are several steps where medical interventions can be made to inhibit fibroblast proliferation, scar formation, and/or subconjunctival and bleb fibrosis[14]. Inflammation suppression and fibroblast growth inhibition are two major steps selected for prolonging the functional period of drainage cannula created during the filtration surgery. Since IL-1 blockers inhibit both inflammation and fibroblast proliferation, they probably are the agents of choice for improving the success rate of filtration surgery in the glaucoma treatment[14,15].

CK-119 and CK-122 are potent anti-inflammatory agents to suppress posterior uveitis induced by intravitreal injection of IL-1[13]. Therefore, these CK-compounds could improve the filtration surgery success rate through their anti-inflammatory actions

CK-119 and CK-122 were also found to have potent inhibitory actions on fibroblast-like corneal and conjunctival cells in this study. These compounds were 10 times more potent to inhibit conjunctival cells than corneal cells. For the purpose of improving filtration surgery, the drugs are given with subtenon's injection to inhibit fibroblast-like conjunctival cells rather than corneal cells. Therefore, drugs more potent to conjunctival cells than corneal cells are much more useful as the effective doses given can act at conjunctiva without affecting corneal cells.

The mechanisms of cell growth inhibition by CK-119 and CK-122 seem to be related to the inhibition of DNA and RNA syntheses and are not related to protein synthesis inhibition in the fibroblast-like corneal and conjunctival cells.

In conclusion, CK-119 and CK-122 are potent agents to inhibit inflammation[13] as well as to suppress cell growth of fibroblast-like corneal and conjunctival cells. Therefore, they could be used to improve the success rate of drainage cannula created by filtration surgery in narrow-angle and/or close-angle glaucomas.

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白细胞介素-1 阻滞剂 CK-119 和 CK-122
对成纤维样细胞增生的抑制

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关键词 哒嗪类；角膜；结膜；培养的细胞；白细胞介素-1；成纤维细胞；细胞分裂；DNA；RNA；蛋白质