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*Original articles*

# Effects of sivelestat, a new elastase inhibitor, on IL-8 and MCP-1 production from stimulated human alveolar epithelial type II cells

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**Abstract**

*Purpose.* The alveolar epithelial cell type II (AEC-II) is itself able to amplify lung inflammation by producing inflammatory cytokines and chemokines, leading to the activation and recruitment of phagocytes. Sivelestat, a new neutrophil elastase inhibitor, has been shown to attenuate acute lung injury in animal experiments. In the current study, we assessed the effects of sivelestat on the production of chemokines from cultured A549 cells, a human AEC-II-like cell line.
*Methods.* A549 cells were stimulated with endotoxin or tumor necrosis factor-α in the presence of sivelestat (1–100 μg·ml$^{-1}$). Culture supernatant levels of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) were determined by enzyme-linked immunosorbent assay. The expression of IL-8 and MCP-1 mRNAs in stimulated A549 cells in the presence of sivelestat (100 μg·ml$^{-1}$) was quantified by real-time polymerase chain reaction.
*Results.* Sivelestat, at 100 μg·ml$^{-1}$ reduced the accumulation of IL-8 and MCP-1 in the culture medium. The high dose of sivelestat significantly inhibited the expression of IL-8 mRNA in A549 cells. The drug also decreased MCP-1 mRNA expression, although not significantly.
*Conclusion.* These data suggest that a high dose of sivelestat regulates the production of IL-8 and MCP-1 in AEC-II.

**Key words** Pneumocytes · Chemokine · Elastase inhibitors · Real-time PCR · Messenger RNA

## Introduction

The alveolar epithelial cell type II (AEC-II) is damaged by many cellular and humoral inflammatory mediators in the development and progression of inflammatory-induced lung injury arising from sepsis, pneumonia, aspiration, and shock. AEC-II is thought to play an important role in the pathogenesis of acute lung injury (ALI) by producing many types of inflammatory mediators [1]. Much attention has recently been focused on the pathophysiological importance of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) in the process of ALI [2,3]. AEC-II is known to release these chemokines [4–6]. In several animal experiments, sivelestat, a new neutrophil elastase inhibitor, has been demonstrated to attenuate acute lung histological damage [7–9]. In addition, sivelestat has been shown to reduce lung production of inflammatory cytokines and chemokines [10,11]. However, the reports have not specified which cell in the lung is a target for sivelestat. Because there is, as yet, no research concerning the effects of sivelestat on the production of the chemokines from AEC-II, we conducted the current study to examine whether sivelestat can inhibit the biosynthesis of IL-8 and MCP-1 in stimulated A549 cells, a human AEC-II-like cell line. To test this, we measured the secretion of these proteins into culture medium, using an enzyme-linked immunosorbent assay (ELISA). The second aim of the current study was to determine the principal site of action of sivelestat by measuring the mRNA expression of IL-8 and MCP-1, if an inhibitory effect of the drug was found in the first experiment for the assessment of protein release.

## Materials and methods

### Cell culture

A549 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) with 10% heat-inactivated fetal calf serum (Sigma), 50 units·ml$^{-1}$ penicillin, 50 μg·ml$^{-1}$ streptomycin (Invitrogen, Carlsbad, CA, USA), and 2.5 μg·ml$^{-1}$ amphotericin B (Sigma).

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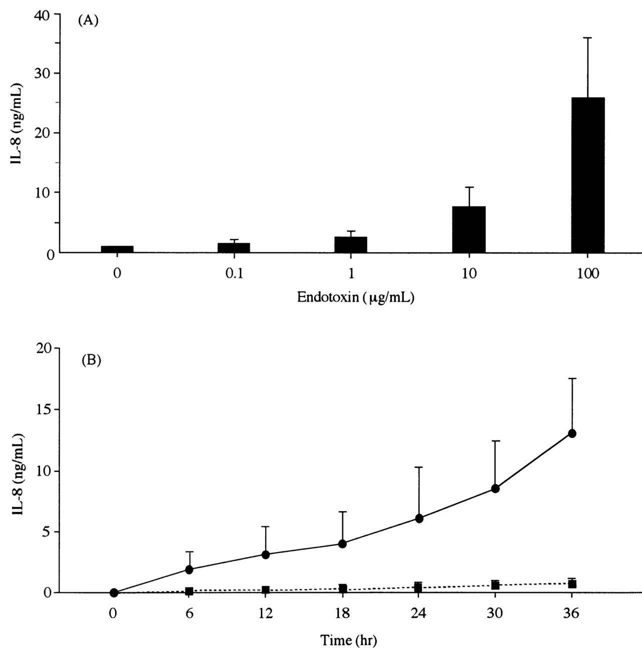


**Fig. 1A,B.** Dose- and time-response analysis of interleukin-8 (*IL-8*) biosynthesis in A549 cells. Data values are given as means ± SD ($n$ = 4 each). **A** Dose-response for endotoxin-mediated IL-8 secretion at 24 h, showing an excitatory effect at a dose of 1 μg·ml$^{-1}$, and achieving the highest level at 100 μg·ml$^{-1}$. **B** Time-response curve for endotoxin (10 μg·ml$^{-1}$)-mediated IL-8 secretion, showing an excitatory effect as early as 6 h, and continuing to increase until 36 h. Concentrations of chemokines in the culture medium were determined with an enzyme-linked immunosorbent assay (ELISA) kit. From the dose-response and time-course data, we assessed the effect of sivelestat on the production of IL-8 by A549 cells stimulated with endotoxin 10 μg·ml$^{-1}$ for 24 h. *Closed squares*, unstimulated A549 ($n$ = 2 each); *Closed circles*, stimulated A549 ($n$ = 4 each)

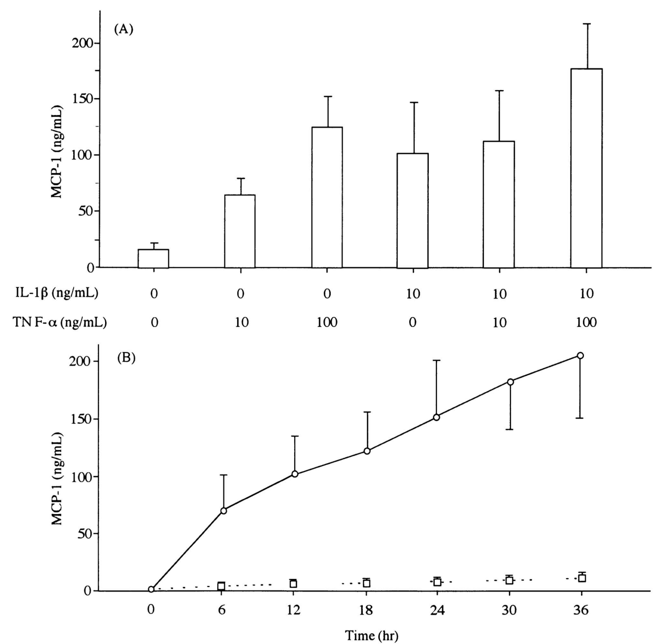


**Fig. 2A,B.** Dose- and time-response analysis of monocyte chemoattractant protein 1 (*MCP-1*) biosynthesis in A549 cells. Data values are given as means ± SD ($n$ = 4 each). **A** Dose-response for tumor necrosis factor α (TNF-α); and/or IL-1β-mediated MCP-1 secretion at 24 h, showing an excitatory effect with TNF-α 10 ng·ml$^{-1}$, and achieving the highest level with a combination of TNF-α 100 ng·ml$^{-1}$ and IL-1β 10 ng·ml$^{-1}$. **B** Time-response curve for TNF-α (100 ng·ml$^{-1}$)-mediated MCP-1 secretion, showing an excitatory effect as early as 6 h, and continuing to increase until 36 h. Concentrations of chemokines in the culture medium were determined with an ELISA kit. From the dose-response and time-course data, we assessed the effect of sivelestat on the production of MCP-1 from A549 cells stimulated with TNF-α 100 ng·ml$^{-1}$ for 24 h. *Open squares*, unstimulated A549 ($n$ = 2 each); *open circles*, stimulated A549 ($n$ = 4 each)

*Dose- and time-response curves for IL-8 and MCP-1 secretion from stimulated A549 cells*

For IL-8 secretion, A549 cells were treated with various concentrations of endotoxin (0–100 μg·ml$^{-1}$; from *Escherichia coli*, serotype 055:B5; Sigma) (see Fig. 1A). For MCP-1 release, the cells were stimulated with a combination of various concentrations of tumor necrosis factor-α (TNF-α; 0–100 ng·ml$^{-1}$; Sigma) and interleukin-1β (IL-1β; 0–10 ng·ml$^{-1}$; Sigma) (see Fig. 2A). The cell-free supernatants were collected 24 h after stimulation and subsequently stored at –80°C until assay. IL-8 and MCP-1 concentrations in the culture supernatant were measured using commercial ELISA kits (human IL-8, RPN2764; human MCP-1, RPN2769; Amersham Biosciences, Buckinghamshire, UK). To assess time-dependent responses of IL-8 and MCP-1, the optimal concentrations of the stimulants (endotoxin 10 μg·ml$^{-1}$ for IL-8 production, and TNF-α 100 ng·ml$^{-1}$ for MCP-1 production; see Figs. 1B and 2B) that caused submaximal induction were subsequently used. A549 cells were challenged with the stimulants for the durations indicated in Fig. 2 (0–36 h). In the preliminary experiments, we confirmed that the stimulation of A549 cells for 24 h increased IL-8 and MCP-1 secretion sufficiently to detect any inhibitory effect of sivelestat (Figs. 1B and 2B).

*Effects of sivelestat on endotoxin- or TNF-α-induced biosynthesis of IL-8 and MCP-1*

*Determination of protein secretion*

For the assessment of the modulatory effect of sivelestat on the biosynthesis of these chemokines, the cells were cultured in six-well plates, at 10$^5$ cells·well$^{-1}$ for 24 h, to 70% confluence; then the medium was replaced with fresh medium containing various concentrations of

sivelestat (0, 1, 10, and 100 μg·ml$^{-1}$; Elaspol, Ono, Osaka, Japan) and the stimulants (endotoxin 10 μg·ml$^{-1}$ for IL-8 production, and TNF-α 100 ng·ml$^{-1}$ for MCP-1 production). These concentrations of sivelestat corresponded to the clinical plasma concentration, and to 10 and 100 times this concentration. After incubation of A549 cells with the stimulants and various concentrations of sivelestat for 24 h, IL-8 and MCP-1 released into the culture medium were measured with the ELISA kits. Even a high dose of sivelestat did not exhibit cell toxicity, as assessed by microscopic cell count 24 h after incubation (data not shown).

*Quantitation of IL-8 and MCP-1 mRNAs*

To assess the time-response of mRNA expression for IL-8 and MCP-1, the cultured A549 cells were used after incubation with the stimulants for specified times (0–24 h), as indicated in Fig. 3. From the time-course of mRNA synthesis, determined by real-time quantitative polymerase chain reaction (PCR), the peak induction of IL-8 and MCP-1 mRNAs occurred at 2 h and 4 h after stimulation, respectively (Fig. 3). Chemokine secretion analysis, using ELISA, revealed that sivelestat, only at 100 μg·ml$^{-1}$, reduced IL-8 and MCP-1 production from stimulated A549 cells, whereas the lower doses of the drug failed to do so (see "Results"). Thus, the effect of sivelestat (100 μg·ml$^{-1}$) on the expression of IL-8 and MCP-1 mRNAs was examied. As shown in Figs. 1–3, there was no parallel between the time-courses of the protein production and mRNA expression in A549 cells. This contradiction can be explained by our methodology. The expression of IL-8 and MCP-1 mRNAs, quantified by real-time PCR, reached its peak within 2–4 h after stimulation and decreased subsequently, but was still found 24 h poststimulation. On the other hand, because the production of IL-8 and MCP-1 proteins was assessed by their accumulated concentrations in the culture medium, but was not assessed by intracellular protein expression using Western blotting, the production of the chemokines showed upward curves.

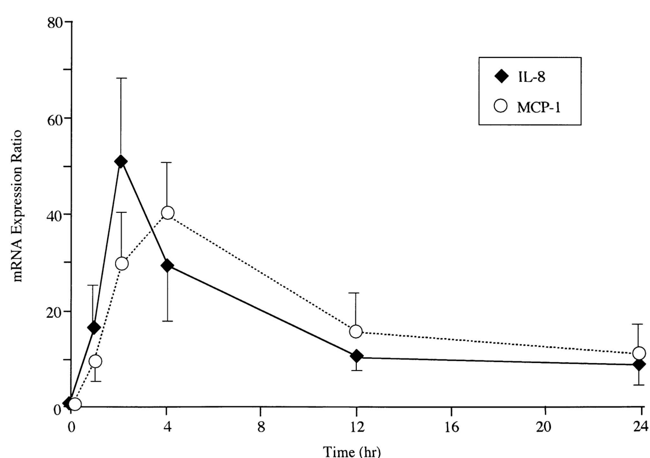


**Fig. 3.** Time-course of mRNA expression for IL-8 and MCP-1 in stimulated A549 cells (mean ± SD; $n = 2$ each). A549 cells were stimulated with endotoxin (10 μg·ml$^{-1}$; for IL-8 production) or TNF-α (100 ng·ml$^{-1}$; for MCP-1 production). The expression of IL-8 and MCP-1 mRNAs was quantified by real-time polymerase chain reaction (PCR)

*RNA extraction and complementary DNA synthesis*

After the incubation of A549 cells (1 × 10$^{6}$) with the stimulants and sivelestat 100 μg·ml$^{-1}$ for 2 h or 4 h, total RNA was extracted from the cells, using a commercial kit (RNeasy Mini Kit; QIAGEN, Hilden, Germany) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis. Four μg of total RNA was reverse transcribed into complementary DNA, in a volume of 12 μl, with Moloney murine leukemia virus reverse transcriptase (SuperScript II; Invitrogen), and random hexamers.

*Quantitative real-time PCR*

Primers and probes for IL-8 and MCP-1 mRNAs were purchased from Applied Biosystems (human IL-8: 4327042F and human MCP-1: 4329524T; Applied Biosystems, Foster City, CA, USA). The mRNA expression of IL-8 and MCP-1 was determined by real-time quantitative PCR, based on TaqMan methodology, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 4326317E; Applied Biosystems) was used for correcting the results by the comparative threshold (CT) cycle method for relative quantification, as described by the manufacturer. The differences between the CT values of the target and GAPDH were calculated (DeltaCT). Relative expression levels were calculated according to the following formula: DeltaDeltaCT = DeltaCT (sample stimulated) – DeltaCT (sample unstimulated), and the value used to plot relative expression was calculated using the expression 2 – DeltaDeltaCT. Dissociation analysis for each primer pair and reaction was performed to verify specific amplification.

*Statistics*

Data values were expressed as means ± SD ($n = 8$ and $n = 5$ for ELISA and real-time PCR, respectively), and were analyzed for statistical significance by one-way analysis of variance, followed by the Student-Newman-Keuls test for post-hoc comparison. $P < 0.05$ was deemed significant.

## Results

### *IL-8*

Sivelestat had no effect on spontaneous IL-8 production in unstimulated A549 cells (data not shown). The endotoxin-induced increase in supernatant IL-8 concentrations was attenuated by 59% with sivelestat at 100 μg·ml$^{-1}$, but was not attenuated at 1 or 10 μg·ml$^{-1}$ (Fig. 4). Sivelestat at 100 μg·ml$^{-1}$ inhibited the endotoxin-induced IL-8 mRNA expression in A549 cells (Fig. 5).

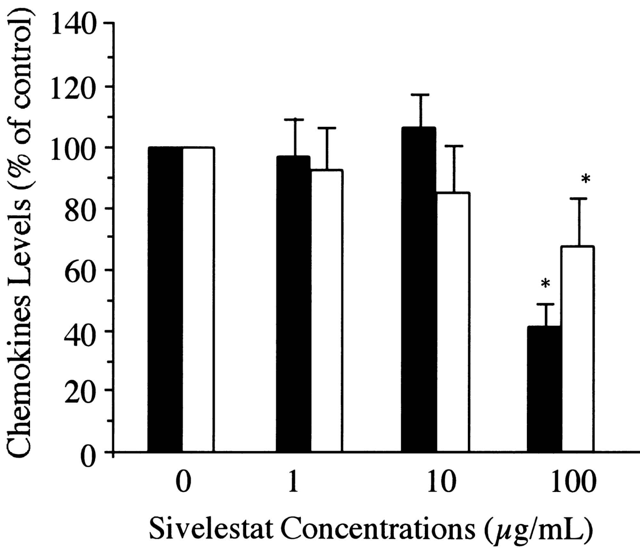


**Fig. 4.** Effects of sivelestat on IL-8 and MCP-1 secretion from stimulated A549 cells. Data values (mean ± SD; $n = 8$ each) are percentages of the control (in the absence of sivelestat). A549 cells were stimulated with endotoxin 10 μg·ml$^{-1}$ or TNF-α 100 ng·ml$^{-1}$ for 24 h. Concentrations of IL-8 and MCP-1 in the culture supernatant were measured with commercial ELISA kits. *$P < 0.05$ vs in the absence of sivelestat (control). *Closed columns*, IL-8; *open columns*, MCP-1

### *MCP-1*

Sivelestat had no effect on spontaneous MCP-1 production in unstimulated A549 cells (data not shown). A high dose of sivelestat reduced TNF-α-stimulated MCP-1 extracellular accumulation (by 33%), whereas the lower doses of the drug failed to reduce the level of MCP-1 (Fig. 4). Sivelestat at 100 μg·ml$^{-1}$ suppressed the TNF-α-induced MCP-1 mRNA expression, although not significantly (Fig. 5).

## Discussion

In the current study, we confirmed previously published findings that AEC-II (A549) cells are capable of producing IL-8 and MCP-1 in response to endotoxin/TNF-α stimulation. Furthermore, we have shown, for the first time, that sivelestat downregulated the biosynthesis of these chemokines from activated A549 cells. We used a cell-culture system which did not contain neutrophils or elastase activity, and AEC-II is unable to produce elastase. In the current study, we could not detect any elastase activity in the culture medium before or after stimulation of A549 cells (data not shown). Thus, the inhibitory effect of sivelestat could be ascribed to its physiochemical characteristics (e.g., lipid solubility) rather than its anti-elastase activity. Because of its lipid-soluble characteristic, sivelestat may change the conformation of receptors, and subsequently may block the

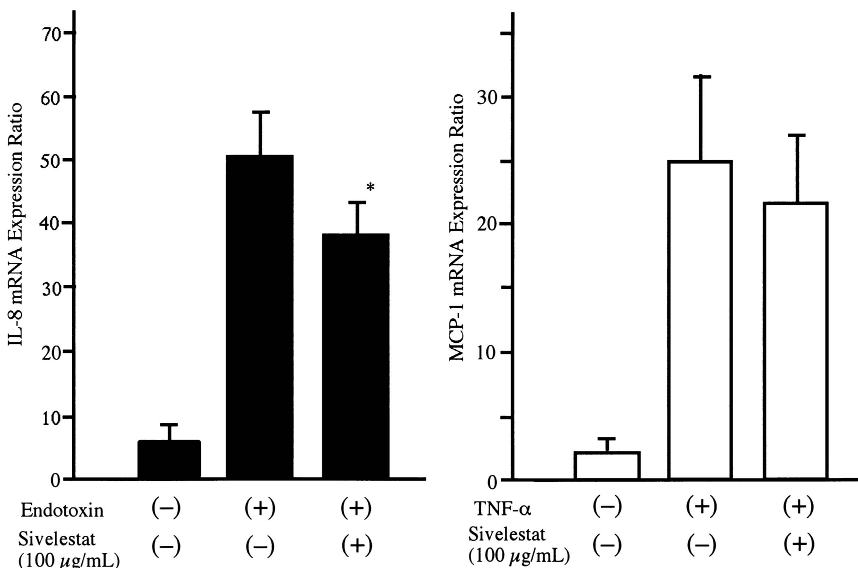


**Fig. 5.** Effects of sivelestat on mRNA expression of IL-8 and MCP-1 in stimulated A549 cells (mean ± SD; $n = 5$ each). A549 cells were incubated with endotoxin 10 μg·ml$^{-1}$ for 2 h (for IL-8 mRNA expression), or with TNF-α 100 ng·ml$^{-1}$ (for MCP-1 mRNA expression) for 4 h in the absence and presence of sivelestat (100 μg·ml$^{-1}$). The expression of IL-8 and MCP-1 mRNAs in A549 cells was analyzed using real-time quantitative PCR. The mRNA expression at time zero was defined as 1. *$P < 0.05$ vs in the absence of sivelestat. *Closed columns*, IL-8; *open columns*, MCP-1

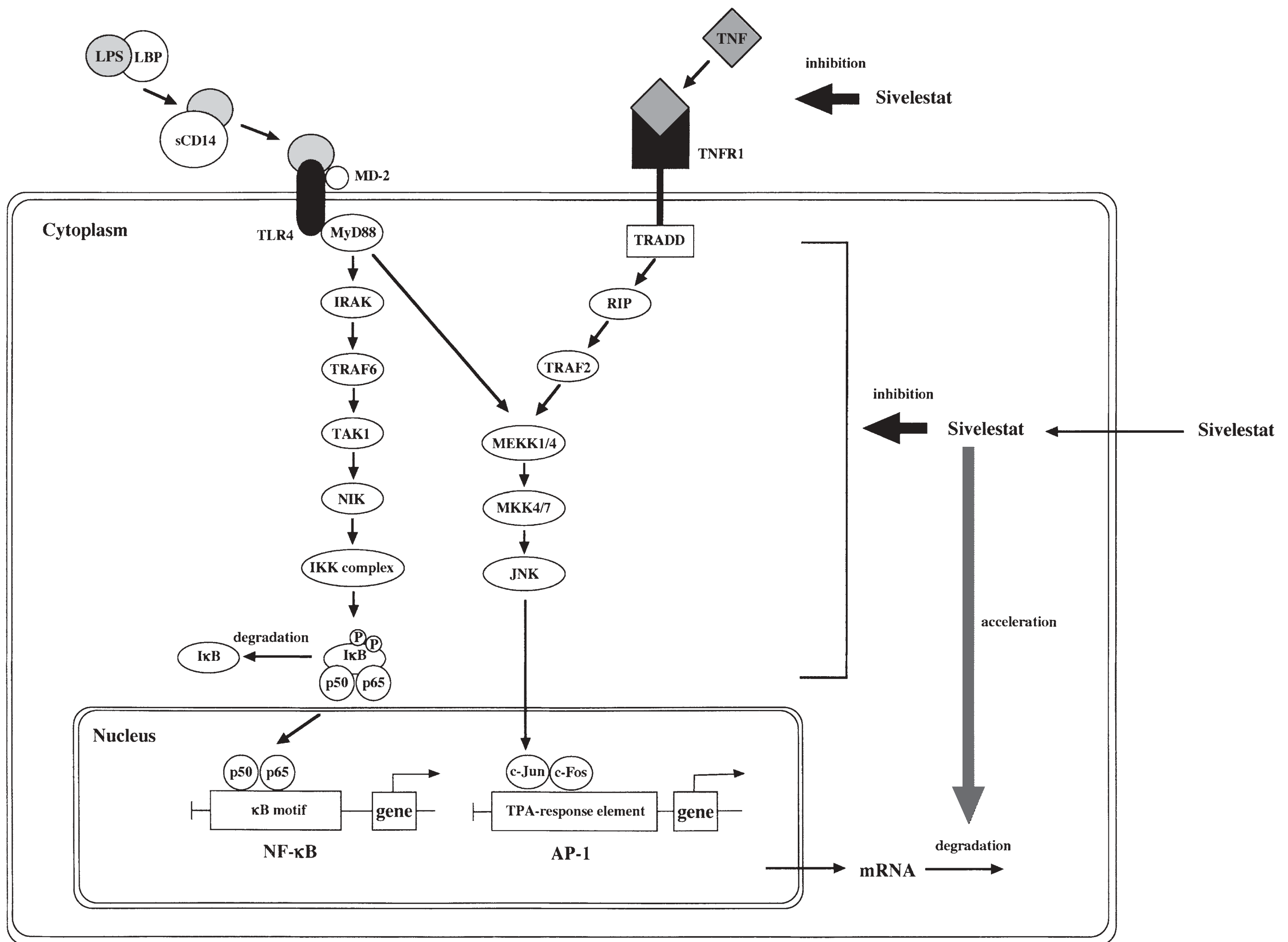


**Fig. 6.** Simplified model of lipopolysaccharide (*LPS*) and TNF signal transduction pathways to induce chemokines. Binding of LPS and TNF-α to their receptors activates many sequential signal factors, finally inducing mRNA expression. Sivelestat may inhibit some steps of the pathways. *sCD14*, soluble CD14; *LBP*, lipopolysaccharide binding protein; *MyD88*, myeloid differentiation factor 88; *TLR4*, Toll-like receptor 4; *IRAK*, IL-1 receptor aassociated kinase; *TAK*, transforming growth factor beta-activated kinase; *NIK*, NF-κB-inducing kinase; *IKK*, IκB kinase; *NF-κB*, nuclear factor κB; *TNFR*, TNF receptor; *TRADD*, TNF receptor-associated death-domain protein; *RIP*, receptor-interacting protein; *TRAF*, TNF receptor-associated factor; *MEKK*, mitogen-activated protein kinase kinase kinase; *MKK*, mitogen-activated protein kinase kinase; *JNK*, Jun N-terminal kinase; *AP-1*, activating protein 1

transduction of intracellular signaling for the gene expression of chemokines (Fig. 6). Furthermore, the drug has the potential to influence signal transduction cascades by reducing intracellular calcium ion influx and by controlling phospholipase C activity [12]. Sivelestat is likely to bind endotoxin or to affect the action of endotoxin-binding protein, thereby inhibiting the binding of endotoxin to Toll-like receptor [13]. Furthermore, sivelestat, which can pass through the cell membrane [14], is possibly able to increase mRNA instability. Besides the data on A549 cells, in vitro data are also available concerning the inhibitory effect of sivelestat on the production of cytokines in culture systems in the absence of neutrophils. Sivelestat inhibits TNF-α production by endotoxin-stimulated peritoneal macrophages isolated from rats with experimental pancreatitis [15]. Sivelestat, at clinically available concentrations, inhibits the endotoxin-induced production of inflammatory cytokines (IL-1β, IL-6, and TNF-α) by human monocytes isolated from healthy volunteers [16]. The data from these experiments may support our speculation that sivelestat can act through mechanisms unrelated to elastase inhibition. However, because we did not assess the effects of sivelestat on the intracellular signal transduction pathway in the current study, the precise mechanism underlying the sivelestat-induced suppression of IL-8 and MCP-1 remains unknown.

Several in vivo studies on the suppression of cytokine production by sivelestat have been published. The drug attenuates both the elevation of serum TNF-α and IL-1β in mice receiving endotoxin [17] and the increase in serum MCP-1 levels associated with liver ischemia-reperfusion in rats [12]. Sivelestat suppresses the increase in blood levels of IL-6 and IL-8 produced after cardiopulmonary bypass in mongrel dogs [18]. The drug also reduces the mRNA expression of inflammatory cytokines (e.g., IL-1β) in the lungs of rats receiving bleomycin [11]. Many cellular and humoral inflammatory mediators constitute the complex network that leads to acute inflammatory conditions, including ALI. Sivelestat is thought to interrupt the vicious circle of the inflammatory mediator network by inhibiting the activity of neutrophil elastase.

In animal in vivo research, sivelestat has been shown to decrease pulmonary leukosequestration in ALI. Sivelestat attenuates endotoxin-induced neutrophil accumulation in the lungs of sheep [13]. The drug also decreases neutrophil counts in bronchoalveolar lavage (BAL) fluid obtained from rats exposed to high concentrations of oxygen [19], and in BAL fluid obtained from guinea pigs receiving endotoxin [20]. However, we found that clinically relevant concentrations of sivelestat failed to decrease the production of IL-8 in A549 cells; thus, the beneficial effect of sivelestat on lung leukosequestration seems to be unrelated to the drug's direct inhibition of chemokine secretion from AEC-II.

The current study has several limitations. First, we used transformed AEC-II (A549 cells). The A549 cell line was initiated in 1972 by Giard et al. [21], through an explant culture of carcinomatous lung tissue from a 58-year-old Caucasian man. Similar to normal AEC-II, A549 cells can synthesize lecithin with a high percentage of desaturated fatty acids [22]. A549 cells have been used in various investigations as a model for the in vitro characterization of human AEC-II functions [23–25]. However, it is likely that A549 cells control the production of inflammatory mediators in a manner different from that of normal AEC-II [26]. Thus, we are unable to simply apply the results on cytokine production found in the current study using A549 cells to cellular responses in normal AEC-II. Secondly, because only a high dose of sivelestat inhibited the production of the chemokines studied, the clinical implication of our findings seems to be low. However, the exposure of AEC-II to high concentrations of sivelestat may be feasible, with the use of an aerosolized form of the drug. The volume of epithelial lung fluid (ELF) in humans is estimated to be approximately 20 ml. An aerosolized form of sivelestat 100 mg, which is used as the standard intravenous dosage, is theoretically expected to increase sivelestat concentrations in ELF to a level as high as $1.5\,mg \cdot ml^{-1}$, provided that 30% of the aerosolized drug is deposited in the alveoli. Thirdly, we should have quantified GAPDH mRNA to exclude the possibility that our results were due to a nonspecific inhibition of mRNA transcription by sivelestat. The absolute amount of GAPDH mRNA is thought to have been unchanged by sivelestat, as the yield of total RNA was similar in the presence and absence of sivelestat (approximately 8.2 μg). A similar amount (4 μg) of total RNA was used for reverse transcription. Furthermore, a similar amount of DNA was used for real-time PCR. The time-cycle (TC), determined using a fixed threshold, was similar for the sivelestat and non-sivelestat groups (mean TC, 18.2 cycles and 18.3 cycles in the sivelestat and nonsivelestat groups, respectively). These data suggest that sivelestat failed to suppress nonspecific gene transcription. Finally, we are unable to determine the site at which sivelestat regulated MCP-1 production (i.e., the transcriptional or post-transcriptional level). On the other hand, a high dose of sivelestat inhibited the expression of IL-8 at the transcriptional level, because the drug reduced the production of IL-8 protein and mRNA. The drug also decreased the expression of MCP-1 mRNA, although not significantly. This could be ascribed to an insufficient sample size ($n = 5$) of the experiment (real-time PCR).

In conclusion, we have shown that a high dose of sivelestat down-regulated the endotoxin-induced biosynthesis of IL-8 and the TNF-α-induced MCP-1 production. We emphasize that in vivo conclusions and clinical implications cannot be drawn at this time because our experiment was an in vitro study.

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