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European Journal of Pharmacology 327 (1997) 169–175

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FR167653, a dual inhibitor of interleukin-1 and tumor necrosis factor-α, ameliorates endotoxin-induced shock

Nobuchika Yamamoto [a,*], Fumihiko Sakai [a], Harumi Yamazaki [a], Natsuki Sato [b], Kunio Nakahara [a], Masakuni Okuhara [a]

[a] *Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan*
[b] *Product Development Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan*

Received 25 November 1996; revised 19 March 1997; accepted 21 March 1997

Abstract

Increased production of interleukin-1 and tumor necrosis factor-α (TNF-α) have been implicated in the pathophysiology of a variety of diseases including circulatory shock. The present study evaluated the efficacy of FR167653 (1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-pyridylpyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate), a dual inhibitor of interleukin-1 and TNF-α production, to protect rabbits from the shock and lethality induced by lipopolysaccharide. In this sepsis model, FR167653 at a dose of 0.32 mg/kg per h ameliorated the 7-day mortality from 93% in the placebo group to 47% in the FR167653-treated group and, at doses of 0.10–0.32 mg/kg per h, attenuated the hypotensive response to lipopolysaccharide challenge and returned mean arterial blood pressure to almost normal levels. The increases in plasma interleukin-1 and TNF-α levels evoked by lipopolysaccharide administration were also inhibited by treatment with FR167653, which was efficacious at doses of 0.1–0.32 mg/kg per h. In addition, FR167653 treatment attenuated the increases in plasma creatinine concentrations consistent with renal damage in a dose-dependent manner. These findings suggested that FR167653 has a beneficial potential as a drug for septic shock or multiple organ dysfunction syndrome.

Keywords: Interleukin-1; TNF-α (tumor necrosis factor-α); Septic shock

1. Introduction

Septic shock is a clinical syndrome caused by Gram-negative and Gram-positive bacteria and by fungi. Patients with septic shock suffer hypotension and acute organ failure, which is associated with a high mortality rate (Dinarello, 1991). Endotoxin elicits interleukin-1 and TNF-α (tumor necrosis factor-α) release in animals and human volunteers, and the appearance of these systemic cytokines corresponds with host responses also seen in septic shock (Endres et al., 1989; Hesse et al., 1988; Cannon et al., 1990). Substantial evidence supports the theory that excessive production of endogenous inflammatory mediators such as interleukin-1 and TNF-α contributes to the pathology of septic shock (Dinarello and Wolff, 1993; Nathan and Sporn, 1991; Tracey et al., 1987).

Experimental administration of interleukin-1, TNF-α and endotoxin has been shown to induce shock in several animal models (Alexander et al., 1991; Beutler et al., 1985; Ohlsson et al., 1990; Tracey et al., 1987). Especially, in models of *Escherichia coli*-induced septic shock, a specific interleukin-1 receptor antagonist, an anti-TNF-α antibody and a soluble TNF receptor effectively ameliorated hypotension, tissue damage and lethality (Wakabayashi et al., 1991; Beutler et al., 1985).

Unfortunately, recent randomized clinical trials of anti-inflammatory agents directed against the presumed mediators of sepsis, including endotoxin, interleukin-1 and TNF-α, have so far failed to show improved survival (McCloskey et al., 1994; Fisher et al., 1993, 1994). However, the interaction between interleukin-1 and TNF-α is highly complicated, and the sepsis patient population is heterogeneous with regard to risk and underlying disease. Therefore, prevention with either interleukin-1 or TNF-α alone is bound to be ineffective or even dangerous, and requires further clinical study.

We have shown that FR167653 is a potent anti-inflammatory drug which inhibits interleukin-1 and TNF-α production in both in vitro and in vivo models of dissemi-

* Corresponding author. Tel.: (81-298) 47-8611; Fax: (81-298) 47-1536.

PII S0014-2999(97)00073-3

nated intravascular coagulation (Yamamoto et al., 1996). In this study, we evaluated the effect of FR167653 on the development of shock and death induced by lipopolysaccharide in the rabbit sepsis model.

2. Materials and methods

2.1. Materials

FR167653 (1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl) pyrazolo [5,1-*c*] [1,2,4] triazin-2-yl]-2-phenylethanedione sulfate monohydrate) was synthesized in our laboratories. Lipopolysaccharide (from *Escherichia coli* 0111:B4) was purchased from Sigma (St. Louis, MO, USA). Di-*n*-butyl phthalate and olive oil were purchased from Nacali Tesque (Kyoto, Japan). Recombinant human interleukin-1β and TNF-α were purchased from Genzyme (Cambridge, MA, USA). [^{125}I]Interleukin-1β was purchased from Amersham (Arlington Heights, IL, USA).

2.2. Experimental rabbit shock model

The rabbit shock model was prepared according to the method of Wakabayashi et al. (1991) with a minor modification. Female New Zealand white rabbits weighing 4–5 kg were anaesthetized with 3% halothane at a flow rate of 20 l/min. The left and right femoral veins were cannulated for administration of lipopolysaccharide and drugs, respectively. The right femoral artery was cannulated and connected to a pressure transducer (AP601G, Nihon Kohden) for measurement of mean arterial blood pressure. After cannulation, the concentration of halothane was maintained at 1% to the end of the experiments. To cause shock, lipopolysaccharide (1 mg/kg) was infused at a flow rate of 0.5 ml/min for 20 min using an infusion pump (STC-523; TERUMO).

FR167653 was given intravenously as a 10 ml/2 min bolus injection (0.032–3.2 mg/kg) 15 min prior to lipopolysaccharide infusion, and then was continuously infused (0.032–3.2 mg/kg per h) at a rate of 3 ml/h for 4 h, which was the same dose as with bolus injection. The saline-treated groups were given saline intravenously as a 10 ml/2 min bolus injection followed by a constant rate of 3 ml/h for 4 h. Blood samples taken at various times were heparinized and centrifuged at 3000 rpm for 10 min. The samples obtained were stored at −80°C until assayed for interleukin-1, TNF-α and creatinine.

To assess the effect of FR167653 on survival rate, female New Zealand white rabbits weighing 4–5 kg were used. The rabbits were anaesthetized with 3% halothane at a flow rate of 20 l/min when the right and left ear veins were cannulated for administration of FR167653 and lipopolysaccharide. FR167653 was intravenously injected at the same time as lipopolysaccharide at a range of 0.01–0.32 mg/kg per h and constant rate of 3 ml/h for 5 h. Lipopolysaccharide (1 mg/kg) was infused at a flow rate of 0.5 ml/min for 20 min by an infusion pump. The lipopolysaccharide-treated group was injected intravenously with saline instead of FR167653. After administration, the cannulae and the anaesthetization were removed and the rabbits were returned to their home cages. The number of survivors and the conditions were monitored for 7 days. Fifteen rabbits were used in each group.

2.3. Measurement of plasma creatinine, interleukin-1 and TNF-α levels

Plasma creatinine concentrations were quantified spectrophotometrically using a Biochemical analyzer (TBA-20R; Toshiba) after conjugation with picric acid. Plasma interleukin-1 levels were quantitated with a receptor binding assay as described previously (Yamamoto et al., 1996). Briefly, murine Balb/3T3 cells expressing the interleukin-1 receptor were harvested by adding 0.2 mM EDTA and were resuspended in binding buffer (RPMI 1640/5% fetal bovine serum/25 mM HEPES, pH 7.2). Each assay sample contained 1.3×10^6 cells and [^{125}I]interleukin-1β at 0.2 nM in 0.15 ml of binding buffer. Non-specific binding was determined by incubation of 5000 U/ml of unlabeled human interleukin-1β in the assay. Incubation was carried out in duplicate for 4 h at 4°C. A 60 μl aliquot of the assay mixture was layered with 150 μl of an oil mixture (di-*n*-butyl phthalate/olive oil, 8:2) and was centrifuged

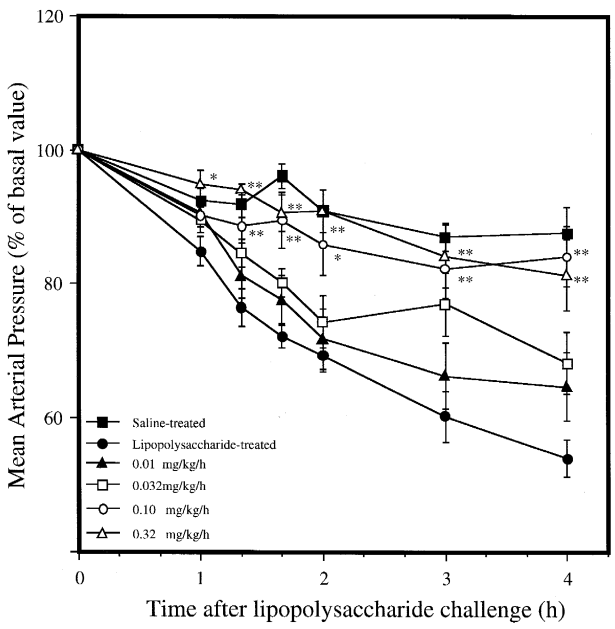

Fig. 1. Effect of FR167653 on mean arterial pressure in anaesthetized rabbits treated with lipopolysaccharide. FR167653 was intravenously injected as a bolus at the doses shown 15 min prior to lipopolysaccharide infusion, then continuously infused for 4 h at the same dose as the bolus injection. The mean arterial pressure values (percent of initial value) are expressed as means ± S.E.M. of 5 observations. Asterisks indicate that the values are significantly different from those of the lipopolysaccharide-treated group: * $P < 0.05$; ** $P < 0.01$.

Table 1
Effect of FR167653 on lipopolysaccharide-induced increases in plasma creatinine levels

Time after lipopolysaccharide challenge (h)	Plasma creatinine levels (mg/dl)				
	Lipopolysaccharide untreated	Lipopolysaccharide-treated			
		Dose of FR167653 (mg/kg per h)			
		0	0.01	0.032	0.10
0	1.31 ± 0.06	1.14 ± 0.06	1.24 ± 0.06	1.23 ± 0.04	1.11 ± 0.02
0.5	1.21 ± 0.08	1.09 ± 0.10	1.21 ± 0.08	1.13 ± 0.05	1.12 ± 0.06
1	1.14 ± 0.05	1.13 ± 0.10	1.21 ± 0.06	1.15 ± 0.04	1.13 ± 0.03
2	1.18 ± 0.07	1.35 ± 0.13	1.39 ± 0.10	1.26 ± 0.04	1.27 ± 0.04
3	1.10 ± 0.14	1.54 ± 0.18	1.54 ± 0.14	1.37 ± 0.04	1.37 ± 0.03
4	1.18 ± 0.10	1.69 ± 0.23	1.65 ± 0.20	1.40 ± 0.06	1.38 ± 0.02
5	1.22 ± 0.10	1.86 ± 0.20	1.74 ± 0.26	1.45 ± 0.06 [a]	1.37 ± 0.06 [a]

Data are presented as means ± S.E.M. for 6 rabbits/group.
[a] $P < 0.05$, significant difference from lipopolysaccharide-treated group at each time point.

for 10 min at 4°C at 15 000 rpm in a microcentrifuge to separate the cell-bound radioactivity from free [^{125}I]interleukin-1β. The tube tip containing the cell pellet was cut off, and the cell-bound radioactivity was determined in a γ-counter. Plasma TNF-α levels were quantitated by L929 cytotoxicity assay. Plasma samples were assayed at a final dilution of 1:100–1:200.

2.4. Statistical analysis of data

The values were expressed as means ± S.E.M. Data from the studies to test the effect of FR167653 on lipopolysaccharide-induced changes in mean arterial pressure, plasma interleukin-1 and TNF-α levels were analyzed with Dunnett's two-tail test. Data from the studies of the effect of FR167653 on prolonging the survival of rabbits challenged with lipopolysaccharide were analyzed with the Peto log-rank test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of FR167653 on mean arterial pressure and creatinine levels in lipopolysaccharide-induced shock

Administration of lipopolysaccharide caused severe hypotension as shown by the decrease in the mean arterial

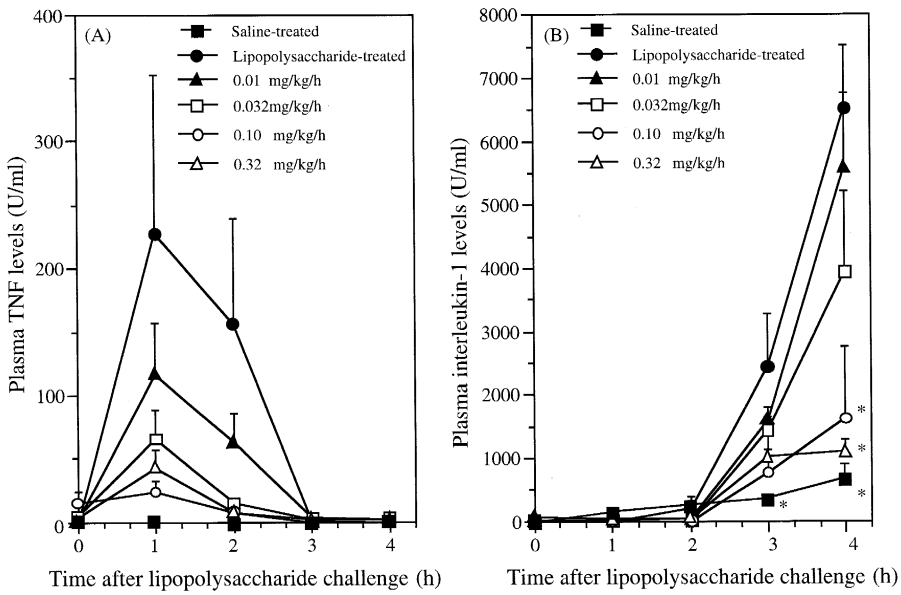

Fig. 2. Effect of FR167653 on lipopolysaccharide-induced increase in plasma interleukin-1 (A) and TNF-α (B) levels in rabbits. FR167653 was intravenously injected as a bolus at the doses shown 15 min prior to lipopolysaccharide challenge followed by continuous infusion for 4 h, at the same dose as the bolus injection. Blood samples were collected from the ear vein at the time points shown. Data are presented as means ± S.E.M. of 5 observations. Asterisks indicate significant difference from the lipopolysaccharide-treated group at the time points: * $P < 0.05$.

pressure (Fig. 1). The average mean arterial pressure started to fall at 1 h after lipopolysaccharide administration and gradually decreased by up to about 54% around 4 h after lipopolysaccharide challenge. Pretreatment with FR167653 at doses of 0.01–0.32 mg/kg per h prevented the hypotension evoked by lipopolysaccharide challenge in a dose-dependent manner. The mean arterial pressure levels in the animals treated with FR167653 at a dose of 0.10 or 0.32 mg/kg per h were almost normal. In the animals treated with placebo, plasma creatinine increased during the first 2 h after lipopolysaccharide administration and then gradually increased up to 5 h (Table 1). The elevation of plasma creatinine levels, which indicates the impairment of renal function, was significantly inhibited by treatment with FR167653 and the inhibitory effect was dependent on the doses of FR167653.

3.2. FR167653 inhibited the elevation of plasma interleukin-1 and TNF-α levels

In this lipopolysaccharide-induced rabbit shock model, different kinetics of interleukin-1 and of TNF-α production were observed (Fig. 2). Plasma TNF-α reached its maximal levels 1 h after lipopolysaccharide challenge and was no longer detectable at 3 h or later. Circulating plasma interleukin-1 levels increased in a time-dependent manner following the peak response of TNF-α and still did not reach a maximal concentration 4 h after lipopolysaccharide challenge. These high levels were markedly reduced in a dose-dependent manner by treatment with FR167653 at doses of 0.01–0.32 mg/kg per h. The degree of inhibition in plasma TNF-α (48.6–89.5%) and interleukin-1 (15.9–92.9%) by treatment with FR167653 was paralleled with the degree of inhibition in mean arterial pressure at 1 h (76.3–132.9%) and 4 h (31.8–89.6%) after lipopolysaccharide challenge, respectively (Figs. 1 and 2).

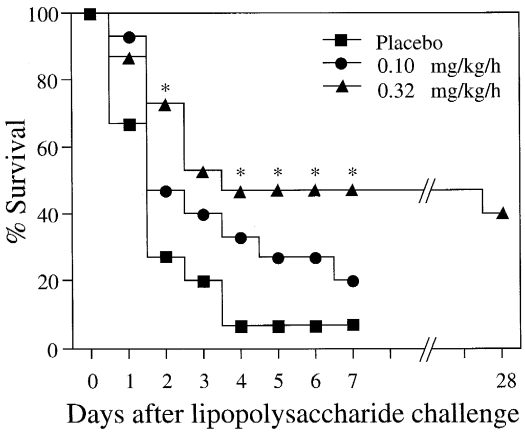

Fig. 3. Effect of FR167653 on survival time in rabbits challenged with a lethal dose of lipopolysaccharide. Administration of FR167653 was started at the same time as lipopolysaccharide infusion (1 mg/kg). FR167653 was infused at a constant rate of 3 ml/h for 5 h and lipopolysaccharide was infused at a flow rate of 0.5 ml/min for 20 min using an infusion pump. Data are expressed as means ± S.E.M. of 15 observations. Asterisks indicate significant difference from the lipopolysaccharide-treated group at these time points: * $P < 0.05$.

3.3. Effect of FR167653 on prolonged survival in rabbits treated with lipopolysaccharide

Fourteen of the 15 rabbits pretreated with placebo died within 1–4 days after lipopolysaccharide challenge (1 mg/kg, intravenous infusion), but one rabbit (7%) survived for 7 days (Fig. 3). In contrast, three of the 15 animals treated with FR167653 0.1 mg/kg per h survived for 7 days. In the group receiving 0.32 mg/kg per h of FR167653, seven of 15 animals survived for 7 days and six of the seven animals survived and appeared in good condition at 28 days after lipopolysaccharide challenge.

4. Discussion

In patients with septic shock, blood interleukin-1 and TNF-α levels could be detected up to 10 days after the onset of shock in a non-survivor, and remained detectable during the state of shock (Calandra et al., 1990; Gardlund et al., 1995; Girardin et al., 1988). Several therapies using anti-cytokine agents such as interleukin-1 receptor antagonist and soluble TNF receptor have been tried but were less effective (Natanson et al., 1994). However, many studies showed that these agents improved survival and organ function in animal endotoxemia models (Alexander et al., 1991; Beutler et al., 1985; Ohlsson et al., 1990; Tracey et al., 1987). Two considerations can account for this discrepancy. First, preventing the elevation of either cytokine alone may be insufficient to reverse septic shock. Interleukin-1 receptor antagonist improved the hypotension induced by *Escherichia coli* in rabbits (Wakabayashi et al., 1991). The inhibitory effect of interleukin-1 receptor antagonist was observed in the late phase of hypotension, which was observed 2–5 h after *Escherichia coli* infusion. However, an early phase of hypotension occurring 0–2 h after *Escherichia coli* infusion was not blocked by interleukin-1 receptor antagonist. Furthermore, administration of interleukin-1 receptor antagonist alone, or of soluble TNF receptor alone, equally and partially prevented the lipopolysaccharide-induced death, whereas soluble TNF receptor was more effective than interleukin-1 receptor antagonist against hepatic and metabolic dysfunction (Russell et al., 1995). Thus, interleukin-1 and TNF-α independently regulated the damaging of several organ functions and hypotension.

Second, interleukin-1 receptor antagonist endogenously produced by infection acted as both a negative and a positive regulator of interleukin-1 production during endotoxemia (Emmet et al., 1996). Transgenic mice producing excess amounts of interleukin-1 receptor antagonist were protected from lethal endotoxin challenge but were more sensitive to infection with *Listeria moocytogenes* than their wild-type control. Serum interleukin-1 levels were higher in the interleukin-1 receptor antagonist overpro-

ducer, than in wild-type mice during endotoxemia: the exogenous administration of interleukin-1 receptor antagonist affected the delicate balance of interleukin-1 levels. Therefore, it is difficult to regulate the balance in a beneficial way and may, in fact, be a drawback in patients with septic shock.

In the present study, we evaluated the effect of FR167653, which inhibits production of interleukin-1 and TNF-α both in vitro and in vivo, in a model of lipopolysaccharide-induced shock in rabbits. FR167653 at doses of 0.01–0.32 mg/kg per h improved lipopolysaccharide-induced hypotension in both the early and the late phases, and suppressed plasma interleukin-1 and TNF-α in rabbits in a dose-dependent manner (Fig. 1). The highest dose of FR167653 did not completely inhibit plasma interleukin-1 and TNF-α, and the remaining plasma cytokines were enough to induce various immune responses. However, the degree of inhibition in plasma interleukin-1 and TNF-α by treatment with FR167653 was consistent with those of inhibition in mean arterial pressure at the maximal levels of the respective cytokines (Figs. 1 and 2). These observation led us to speculate that excess amounts of interleukin-1 and TNF-α may be needed to induce shock states evoked by lipopolysaccharide.

In addition, the inhibitory degree by FR167653 on maximal levels of interleukin-1 was different from that of TNF-α. The difference of inhibition between interleukin-1 and TNF-α was also observed on restoration of mean arterial pressure at each time point that plasma cytokine reached to maximal levels (Figs. 1 and 2). Interleukin-1 receptor antagonist could not prevent the decrease of mean arterial pressure in the early phase, and the pattern of mean arterial pressure decline was paralleled by the increase of plasma TNF-α (Wakabayashi et al., 1991). These observations support the possibility of independence of the actions of interleukin-1 and those of TNF-α against mean arterial pressure.

Moreover, interleukin-1 and TNF-α are known to lead to disseminated intravascular coagulation characterized by microvascular thrombosis in various organs (Okusawa et al., 1988; Dinarello, 1991; Beutler et al., 1985). Disseminated intravascular coagulation is an important pathogenic factor for the development of multiple organ dysfunction and a common feature of lethal septic shock (Carrico et al., 1986; Fourrier et al., 1992). FR167653 showed a suppressive effect against lipopolysaccharide-induced disseminated intravascular coagulation in rats (Yamamoto et al., 1996). Lipopolysaccharide administration causes impairment of renal function, as indicated by an increase in plasma creatinine (Table 1) and FR167653 caused a significant improvement of this parameter. Further, FR167653 at 0.01–0.32 mg/kg per h improved the survival rate of rabbits challenged with lipopolysaccharide (Fig. 3). As mentioned above, FR167653 acted as a protective agent in lipopolysaccharide-induced hypotension and death, and this protective effect may be due to its prevention of the increase in the levels of interleukin-1 and TNF-α, but we cannot eliminate the possibility of some additional actions of FR167653.

Endotoxin in combination with interleukin-1 and TNF-α has been shown to induce the inducible nitric oxide (NO) synthase, which is an endogenous vasodilator that has also been implicated in sepsis-induced hypotension and blood flow abnormality (Cobb and Danner, 1996). However, results of several studies using mice that lack inducible NO synthase suggested that there exists both inducible NO synthase-dependent and inducible NO synthase-independent pathways to lipopolysaccharide-induced hypotension and death. When the mice lacking inducible NO synthase were primed with *Propionobacterium acnes* and challenged with lipopolysaccharide, the mice suffered lipopolysaccharide-induced severe liver damage and hypotension (MacMicking et al., 1995). There is another report that inducible NO synthase-deficient mice receiving a lethal dose of lipopolysaccharide did not exhibit a significantly longer survival time than did wild-type mice (Laubach et al., 1995). These data indicate that absence of inducible NO synthase activity does not sufficiently prevent lipopolysaccharide-induced mortality and tissue damage.

The simultaneous suppression of interleukin-1 and TNF-α has improved survival time and organ dysfunctions in several rodent models of septic shock analogous to those of bacteremia and endotoxemia models and not to systemic inflammatory response syndrome. Although these data suggested that interleukin-1 and TNF-α have pivotal roles in septic shock, it remains unclear whether inhibition of these cytokines will lead to ameliorate the shock states in patients with septic shock.

However, we supposed that the protective effect of FR167653 on survival and on several organ dysfunctions may be more effective than that of interleukin-1 receptor antagonist and soluble TNF-α receptor, because FR167653 suppressed lipopolysaccharide-induced damage to various organ functions and hypotension in animal models elicited independently by interleukin-1 and TNF-α. In addition, since FR167653 is not an antagonist, but an inhibitor of cytokine production, the mechanism for the inhibitory effect of FR167653 is thought to be different from that of interleukin-1 receptor antagonist and may not yield any agonistic responses seen with interleukin-1 receptor antagonist (Emmet et al., 1996).

Therefore, we propose that FR167653 belongs to a novel class of drugs for the treatment of systemic inflammatory response syndrome such as septic shock and multiple organ dysfunction syndrome.

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