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European Journal of Pharmacology 509 (2005) 71–76

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Pharmacological profile of FR260330, a novel orally active inducible nitric oxide synthase inhibitor

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Received 24 June 2004; received in revised form 14 December 2004; accepted 17 December 2004
Available online 29 January 2005

Abstract

In this study, we examined effects of a newly synthesized chemical compound, FR260330, (2*E*)-3-(4-chlorophenyl)-*N*-[(1*S*)-2-oxo-2-{[2-oxo-2-(4-{[6-(trifluoromethyl)-4-pyrimidinyl]oxy}-1-piperidinyl)ethyl]amino}-1-(2-pyridinylmethyl)ethyl]acrylamide on nitric oxide (NO) production in rat splenocytes and human colon cancer cell line, DLD-1 cells. FR260330 inhibited NO*x* production dose dependently in both cells. In lipopolysaccharide (LPS) and interferon-γ (IFN-γ) treated murine macrophage cell line, RAW264.7, Western blot analysis with gel filtration chromatography revealed FR260330 might prevent dimerization of inducible nitric oxide synthase (iNOS), but had no effect on the expression of iNOS protein. Furthermore, oral administration of FR260330 reduced NO*x* production dose dependently in plasma from rats exposed to LPS (IC_{50}=1.6 mg/kg). Meanwhile, higher dose (100 mg/kg) of oral administration of FR260330 did not change mean arterial blood pressure in rats. These results suggest that FR260330 might be a useful therapeutical approach to various inflammatory diseases, in which superoxide or peroxynitrite formed from iNOS-derived NO are involved.

Keywords: Inducible nitric oxide synthase; FR260330; Dimerization

1. Introduction

Nitric oxide (NO) is involved in the regulation of many physiological process, as well as in the pathophysiology of a number of diseases. It is synthesized enzymatically from L-arginine by three distinct isoforms of the enzyme, nitric oxide synthase (NOS).

Two of these isoforms are constitutively expressed, predominantly in the vascular endothelium (eNOS) (Forstermann et al., 1991) and in the nervous system (nNOS) (Ogura et al., 1993). Under normal physiological conditions, these constitutive forms of NOS generate low, transient levels of NO in response to increases in intracellular calcium concentrations (Forstermann et al., 1991). These low levels of NO act to regulate blood pressure (Aisaka et al., 1989), platelet adhesion (Radomski et al., 1987), and neurotransmission (Sanders and Ward, 1992).

The expression of the third isoform (iNOS) is induced by endotoxin and/or cytokines and generate high, sustained levels of NO (Xie et al., 1992). These elevated levels of NO and resulting NO-derived metabolites cause cellular cytotoxicity and tissue damage and are thought to contribute to the pathophysiology of a number of human diseases (Sirsjo et al., 1996; Singer et al., 1996; Dattilo et al., 1997; Furusu et al., 1998).

Non-selective inhibition of NO formation could lead to side effects, since this could lead to the inhibition of the constitutive isoforms of NOS, which is responsible for a number of physiological actions of NO. Based on this scenario, selective inhibitors of iNOS may well have considerable therapeutic potential, particularly if they do not interfere with the physiological roles of eNOS and

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doi:10.1016/j.ejphar.2004.12.028

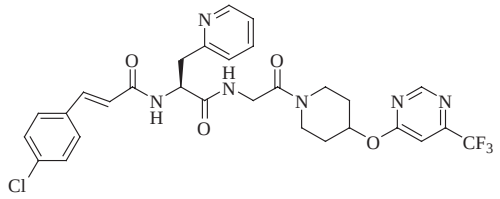

Fig. 1. Chemical structure of FR260330.

nNOS. Therefore, a considerable effort has been made to develop a novel iNOS inhibitor which would be useful as a biological tools and, more importantly, as a possible therapeutic agent for use in the treatment of various diseases.

Here we report a novel and potent iNOS inhibitor, FR260330, (2*E*)-3-(4-chlorophenyl)-*N*-[(1*S*)-2-oxo-2-{[2-oxo-2-(4-{[6-(trifluoromethyl)-4-pyrimidinyl]oxy}-1-piperidinyl)ethyl]amino}-1-(2-pyridinylmethyl)ethyl]acrylamide (Fig. 1), which has potent inhibitory activity, both in vitro and in vivo, and does not inhibit iNOS enzyme activity but may inhibit dimerization of iNOS.

2. Materials and methods

2.1. Compound

The following drugs were used: N_w-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 obtained from Sigma-Aldrich (Tokyo, Japan). L-N^6-(1-Iminoethyl) lysine hydrochloride (L-NIL), *N*-1-naphthylene-diamine dihydrochloride and sulfanilamide were obtained from Wako (Tokyo, Japan). interleukin-1β and tumor necrosis factor-α (TNF-α) were obtained from Genzyme-Techne. Interferon-γ (IFN-γ) was obtained from BD biosciences. [^{14}C] L-arginine was obtained from Amersham Biosciences. FR260330 was chemically synthesized in our laboratories. For in vitro assays, L-NAME, L-NIL and FR260330 were dissolved in dimethyl sulfoxide (DMSO), and further dilutions were made in culture medium. The final concentration of DMSO was less than 0.1%. Other compounds were dissolved in culture medium or phosphate buffered saline (PBS). For in vivo administration, FR260330 was dissolved in polyethyleneglycol (PEG) 400 solution.

2.2. Cell culture

A human colon cancer cell line, DLD-1, and a murine macrophage cell line, RAW264.7, were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% FCS.

For primary splenocytes culture, spleens were removed from rats after decapitation and splenocytes were isolated by trypsin digestion. These cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FCS.

2.3. Animals

The experimental work was reviewed by the Animal Ethical Committee of Fujisawa Pharmaceutical for Animal Experimentation.

Male Lewis rats (8 weeks old) were purchased from Charles River Japan. Animals received food and water ad libitum, and lighting was maintained on a 12-h cycle.

2.4. Endotoxin or cytokine induced NO production in vitro

Spleens of Lewis rats were removed from rats after decapitation and cut into about 10 pieces. The pieces of spleens were levigated with two slide glasses. The levigated spleens were passed through cell strainers and centrifuged. The pellets were flaked and hemolyzed with Tris–NH_4Cl. The hemolyzed cell suspension was centrifuged and then flaked with culture medium. Additionally, the cell suspension was centrifuged and flaked with culture medium. This procedure was repeated three times. The resulting pellets were suspended with culture medium, passed through cell strainers and diluted at 4×10^6 cells/ml. The cell suspension was added to 96 well plates and cultured for 24 h.

DLD-1 cells were cultured at an initial cell density of 1×10^5 cells/well in 96 well plates supplemented with various concentrations of FR260330, 0.5 ng IL-1β/ml, 100 units IFN-γ/ml and 10 ng TNF-α/ml. After 24 h LPS or cytokine stimulation, Griess reagent (1:1 mixture of 0.1% *N*-1-naphtylethylene-diamine dihydrochloride and 1% sulfanilamide) was added in each well and measured $OD_{550/655}$ absorbance density.

2.5. Enzyme assay with iNOS derived from RAW264.7 cells

RAW264.7 cells were cultured in 10% FCS supplemented with 4 μg LPS/ml and 16 U IFN-γ/ml. After 24 h, cells were collected and centrifuged. The pellets were suspended with Tris-buffer containing several protease inhibitors (1 mM dithiothreitol (DTT), 10 μg/ml soybean trypsin inhibitor (SBTI), 10 μg/ml antipain, 10 μg/ml leupeptin and 100 μg/ml pefbloc) and the cell suspension were treated with ultrasonic wave on ice to homogenate cells. The resulting cells were centrifuged at 20,000×*g* for 60 min and the supernatants were used for enzyme assay as enzyme solution. The enzyme solution was pre-incubated with coenzyme (4 mM DTT, 400 μM nitrate oxidoreductase (NADPH), 40 μM flavin adenine dinucleotide (FAD) and 40 μM tetrahydrobiopterin (BH4)) at 37 °C for 15 min. [^{3}H] L-arginine were added and incubated at 37 °C for 60 min. Then ion exchange resin (DOWEX 50W X8) suspended with distilled water were added and incubated for 5 min with shaking. Moreover the reaction mixture was centrifuged at 2000 rpm for 5 min and the

radioactivity in the supernatants was counted using top counter.

Inhibitory activities of test compounds were determined by measuring the conversion rate from [^{14}C] L-arginine to [^{14}C] L-citrulline, and the IC_{50} values were determined.

2.6. Western blot analysis

RAW264.7 cells were grown in DMEM containing 10% FCS with a F75 culture flask. When the cells were grown in confluent, 4 μg LPS/ml and 16 units IFN-γ/ml were added in the medium. After 8 h culture, the cells were harvested by centrifugation, washed twice in PBS and resuspended in lysis buffer. Resulting cell suspension was stored at −20 °C until use. In case of treatment of FR260330, the cells were incubated with this compound at the induction of iNOS. The cells were lysed by three times freeze thawing and the suspension was centrifuged at 10,000×*g* for 60 min at 4 °C. The supernatant was collected and stored at −20 °C until use.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by a conventional procedure using a 4–15% gradient gel. The cell extracts were mixed with SDS treated buffer and electophoresed and transferred onto a polyvinylidene fluoride (PVDF) membrane using an electro-blotting apparatus. The blotted proteins were proved using a rabbit polyclonal antibody raised against mouse macrophage iNOS, followed by detection using electrochemiluminescence (ECL) detection system.

2.7. Gel filtration chromatography

The cell extracts from cells treated with LPS and IFN-γ in the presence or absence of FR260330 were analyzed by size exclusion chromatography followed by measuring NOS activities. The extracts were subjected to Superolex 200HR 10/30 (flow rate; 0.5 ml/min) equilibrated with 25 mM Tris–HCl (pH 7.0) containing 0.15 M NaCl, previously, eluted and fractionated. Aliquots of each fraction were assayed for NOS activities and Western blot analysis.

2.8. Endotoxin-induced NO production in vivo

Male Lewis rats were orally administered with FR260330 (0.1 to 32 mg/kg body weight) followed by intravascular (i.v.) injection with LPS dissolved with saline, 1 mg/kg body weight, 30 min later. Blood was collected 3.5 h after LPS injection. NO production in plasma was assessed by measuring nitrite/nitrate, the stable degradation products of NO, as follows. The plasma was isolated by centirifugation of whole blood at 3000 rpm for 10 min and incubated with co-enzyme (NADPH and FAD) at 37 °C for 15 min. Next, the reaction mixture was incubated with nitrate reductase at 37 °C for 30 min. The Griess reagent was added to the reaction mixture and $OD_{550/655}$ absorbance density was measured.

2.9. Measurement of blood pressure

Male Lewis rats were anesthetized by ether, and the femoral artery was canulated. The rats were placed in restraining cages, and the cannula was connected to a Code disposable pressure transducer. After rats were awaken, hemodynamic parameters were measured under no anesthesia. FR260330 was administered orally by gavage. Mean arterial blood pressure and heart rate were recorded for 6 h following compound administration.

2.10. Statistical analysis

The IC_{50} values were calculated from the dose-percent inhibition relation, using a computerized log-linear regression analysis. And groups stimulated with cytokines or LPS with no drug treatment were assigned for determinations of the zero value for IC_{50} calculations. And the differences of the values of mean arterial blood pressure were analyzed using Dunnett's test.

3. Results

3.1. Inhibition of NO accumulation in culture and mode of action by FR260330

The splenocytes from rats stimulated with LPS, and the human colon cancer cell line, DLD-1, costimulated with IL-1β, TNF-α and IFN-γ, were used as in vitro model and the effect of FR260330 on NO release was determined. FR260330 inhibited NO accumulation in a dose-dependent manner in both cells. The IC_{50} value was 27 nM in rat splenocytes and 10 nM in human DLD-1 cells (Fig. 2).

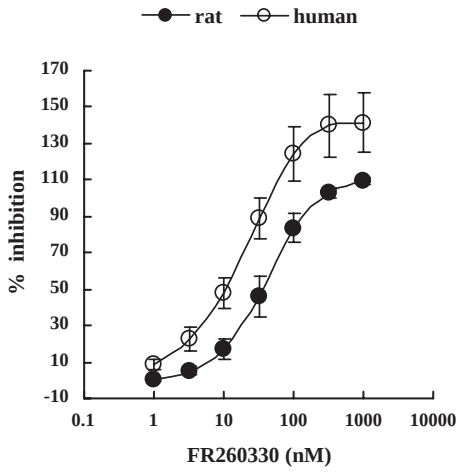

Fig. 2. Inhibition of NO accumulation by FR260330 in rat splenocytes stimulated with LPS (closed circles) and in human DLD-1 cells stimulated with IL-1β, TNF-α and IFN-γ (open circles). FR260330 inhibited NO accumulation in a dose-dependent manner in both cells. Data are expressed as mean±standard error (n=3).

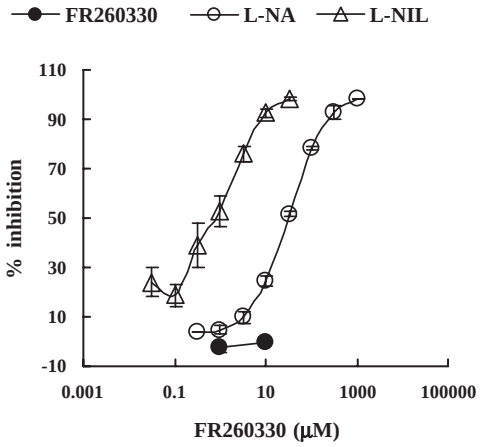

Fig. 3. Direct inhibition of iNOS activity in RAW264.7 cells stimulated with LPS and IFNγ. FR260330 (closed circles) failed to inhibit iNOS activity directly, but L-NAME (open circles) and L-NIL (open triangles) dose dependently inhibited iNOS activity. Data are expressed as mean± standard error (n=3).

Next, the effect of FR260330 on iNOS activity in murine macrophage cell line, RAW264.7, was examined. At 1 μM, FR260330 inhibited NO accumulation completely in this culture medium co-stimulated with LPS and IFN-γ (data not shown). On the other hand, even at 10 μM, FR260330 failed to inhibit iNOS activity, which was prepared from RAW264.7 stimulated with LPS and IFN-γ, while L-NAME and L-NIL dose-dependently inhibited iNOS activity (Fig. 3). This suggested that FR260330 inhibited iNOS activity indirectly.

To investigate further the inhibitory mechanism of FR260330, we studied its effect on the expression of iNOS protein in LPS and IFN-γ stimulated RAW264.7 cells. FR260330 had no effect on the expression of iNOS protein at 1 μM (Fig. 4).

Another possibility was that FR260330 affected iNOS dimerization, which was essential for enzyme activity. The extracts from activated RAW264.7 cells in the presence or absence of FR260330 at 1 μM were fractionated by gel filtration chromatography and the fractions were analyzed by dot blotting using anti-iNOS antibody. In the absence of

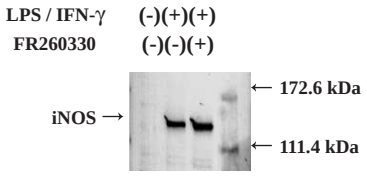

Fig. 4. Western blot analysis of iNOS in RAW264.7 cells stimulated with LPS and IFNγ. From left to right lane, each lane represents the blot derived from no stimulated cells, stimulated cells in the absence of FR260330, stimulated cells in the presence of FR260330 and protein size marker. The arrow in the left side shows the band corresponding to the 130-kDa iNOS protein. These blots are representative from three experiments. The ratio of the density of the blot in the presence of FR260330 stimulated with LPS and IFNγ compared with the density in the absence of FR260330 is 102.3±6.5% <mean±standard error> (n=3).

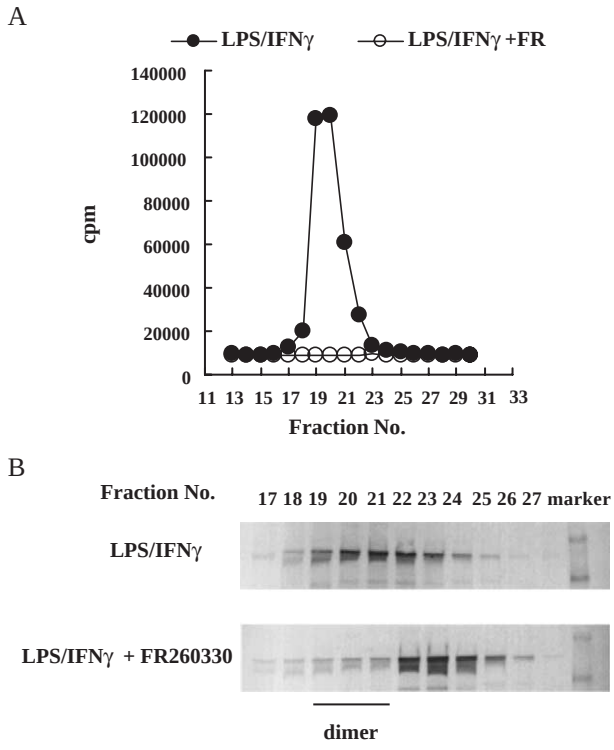

Fig. 5. iNOS activity (A) and expression (B) in the extracts from RAW264.7 cells stimulated with LPS and IFNγ in the presence or absence of FR260330. (A) In the absence of FR260330, iNOS activity was detected in fraction No. 19 to No. 21 (closed circles), but in the presence of FR260330, the activity disappeared (open circles). (B) iNOS protein was detected in fraction No. 19 to No. 23 in the absence of FR260330 (upper photo). In the presence of FR260330, iNOS protein was detected in fraction Nos. 22–25 (below photo), showing no iNOS activity. The data are representative from three experiments.

FR260330, iNOS activity was detected in fraction No. 19 to No. 21, but in the presence of FR260330, the activity disappeared (Fig. 5(A)). From the Western blot analysis, iNOS protein was detected in fraction No. 19 to No. 23 in the absence of FR260330. In the presence of FR260330, iNOS protein was detected in fraction Nos. 22–25, showing no

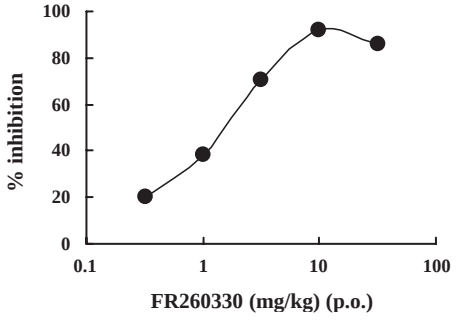

Fig. 6. The inhibitory effect of FR260330 on NO production in rats exposed to LPS (1 mg/kg (i.v.)) (closed circles). FR260330 dose dependently inhibited NO production (IC_{50}=1.6 mg/kg (p.o.)). The NOx value in vehicle treated group 3.5 h after LPS exposure was 131.16±9.75 (μM) <mean±standard error> (n=4).

iNOS activity (Fig. 5(B)). These results indicated that inhibition of NO*x* production by FR260330 in stimulating RAW264.7 cells might be due to prevention of iNOS dimerization, not to a decrease in its de novo synthesis.

3.2. Effect of plasma levels of nitrite/nitrate in LPS-treated rats

To examine the inhibitory effect of FR260330 on NO production in vivo, the plasma level of nitrite/nitrate (NO*x*) was determined in rats exposed to LPS with or without FR260330. Elevations in plasma NO*x* levels had been used as an index of systemic iNOS induction. Oral administration of rats with FR260330 30 min prior to the systemic induction of iNOS by LPS led to dose-dependent inhibition of elevated plasma NO*x* levels (IC_{50}=1.6 mg/kg) measured 3.5 h after LPS administration (Fig. 6).

3.3. Effect on mean blood pressure in non-anethesized rats

To examine whether FR260330 inhibits eNOS in vivo, the effect on mean arterial blood pressure in non-anethesized rats was determined. If this compound had a prolife to inhibit eNOS activity like other non-specific NOS inhibitor, the mean arterial blood pressure was elevated after oral administration with it. But even at a dose of 100 mg/kg (p.o.), there were no significant differences in mean arterial blood pressure compared with vehicle treated group (Fig. 7). So FR260330 selectively inhibited iNOS but not eNOS activity in the experimental conditions.

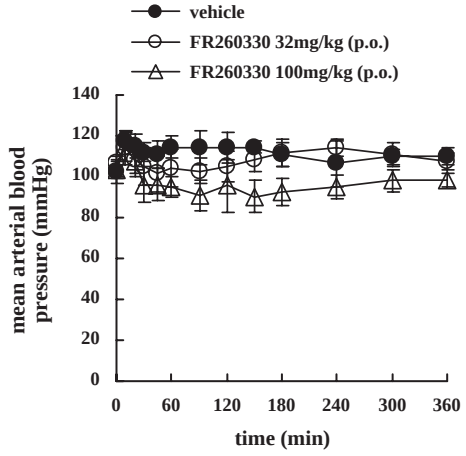

Fig. 7. Effect of FR260330 on mean arterial blood pressure in non-anethesized rats. Closed circles (●), open circles (○) and open triangles (△) indicates the value of vehicle treated group, FR260330 32 mg/kg (p.o.) treated group and FR260330 100 mg/kg (p.o.) treated group, respectively. FR260330 did not elevate mean arterial blood pressure even at 100 mg/kg (p.o.), about 60 times higher than the value of IC_{50} to inhibit NO production in rats exposed to LPS (1 mg/kg (i.v.)). There were no significant differences in mean arterial blood pressure among three groups using Dunnett's test. Data are expressed as mean±standard error (n=4).

4. Discussion

We have demonstrated that the newly synthesized compound FR260330 strongly inhibits iNOS activity by the mechanism hypothesized as reducing dimerization of the monomeric form. The IC_{50} value determined in rat splenocytes, which are rich in macrophages which could induce iNOS stimulated by LPS (Bian and Murad, 2001), was 27 nM and in human colon cancer cell line, DLD-1, which is one of the most famous cell lines derived from human which could induce iNOS stimulated with IL-1β, TNF-α and IFN-γ (Kleinert et al., 1998), was 10 nM. So species difference of iNOS inhibitory activity of FR260330 in vitro between rats and human might be minimal, although sorts of tested cells were different.

FR260330 failed to inhibit the iNOS activity in crude cell lysates, which could contain a lot of iNOS-form homo-dimers, suggesting it might not be able to dissociate the dimer.

It has been reported that a compound with a pyrimidine-imidazole core inhibited NO production by interfering with the homo-dimerization of iNOS (Sennequier et al., 1999). X-ray crystallography revealed that the bulky imidazole residue reacted with the substrate binding site and with the dimerization interface, leading to allosteric disruption of protein–protein interaction at the dimer interface. But FR260330 does not have an imidazole residue, so which site this compound binds on the monomer form remains to be determined.

As expected from the in vitro results, FR260330 also inhibited iNOS activity in LPS-treated rats. In response to LPS, host cells, particularly macrophages, release inflammatory mediators such as TNF-α, IL-1β, IL-6, IFN-γ and NO (Salkowski et al., 1997). Considerable evidence suggests that excessive production of NO by iNOS contributes to the circulatory failure observed during septic shock, and iNOS inhibitors prevent LPS-induced mortality in mice (Szabo et al., 1994) and iNOS dimerization inhibitor can prevent cardiovascular and renal morbidity in sheep (Enkhbaatar et al., 2003). Oral administration of FR260330 decreased LPS-induced nitrite/nitrate levels in plasma, indicating that FR260330 was biologically active in vivo. Endogenous IFN-γ production has been demonstrated to be a key step in the LPS-mediated induction of iNOS mRNA and in the accumulation of serum nitrite/nitrate (Salkowski et al., 1997). However, FR260330 did not inhibit IFN-γ production in rat mononuclear cells stimulated with LPS or concanavalin A (Con A) in vitro (data not shown), indicating that this compound inhibited in vivo iNOS activity directly, not through an indirect mechanism such as decreasing the production of LPS-inducible cytokines.

We also demonstrated that high dose (100 mg/kg) of oral administration with FR260330 did not affect mean arterial blood pressure in rats, indicating even a high dose of this compound might not inhibit eNOS activity in vivo. This might be due to the isoform specificity for inhibition of

dimerization for NOS, because the energetics and kinetics for monomer–dimer equilibra of iNOS have been thought much fragiler than eNOS (Blasko et al., 2002). On the other hand, it has been reported that non-specific NOS inhibitors increase mean arterial blood pressure by inhibiting eNOS activity in vivo (Prado et al., 1992; Takahashi et al., 1992; el Karib et al., 1993). Therefore, in clinical and in pre-clinical studies, non-specific NOS inhibitors could not be useful. FR260330 might have an advantage not affecting mean arterial blood pressure at the close dose to inhibit iNOS in vivo over other non-specific NOS inhibitors.

In conclusion, a newly synthesized non-imidazole compound, FR260330, inhibited the production of NO*x* by rat splenocytes, human colon cancer cell line, DLD-1, and murine macrophage cell line, RAW264.7, stimulated with LPS and/or cytokines. Inhibition of NO*x* production by FR260330 might be due to prevention of iNOS dimerization, an essential key step for enzyme activity. The inhibitory effect of FR260330 was also observed in rats treated with LPS in vivo. High dose (100 mg/kg) of FR260330 did not change mean arterial blood pressure in rats. Therefore, FR260330 might be a useful therapeutical approach in pre-clinical and in clinical studies to examine the role of not only NO but also superoxide or peroxynitrite formed from iNOS-derived NO in many inflammatory and auto-immune diseases.

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