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Discovery of novel inhibitors of inducible nitric oxide synthase

Yasuhiro Kita, Masakazu Muramoto, Akihiko Fujikawa, Takao Yamazaki, Yoshitada Notsu and Shintaro Nishimura

Abstract

We have discovered three compounds, 5-chloro-1,3-dihydro-2H-benzimidazol-2-one (FR038251), 1,3(2H,4H)-isoquinolinedione (FR038470) and 5-chloro-2,4(1H,3H)-quinazolonedione (FR191863), which show inhibition of inducible nitric oxide synthase (iNOS). The iNOS inhibitory activity of the compounds was examined in comparison with that of aminoguanidine, a representative iNOS inhibitor. FR038251, FR038470 and FR191863 inhibited mouse iNOS, with IC50 values of 1.7, 8.8 and 1.9 μM, respectively, in an in-vitro enzyme assay. These inhibitory activities are comparable with that of aminoguanidine (IC50 = 2.1 μM). The three compounds had iNOS selectivity 38- and 8-times, >11- and 3-times, and 53- and 3-times compared with rat neuronal NOS and bovine endothelial NOS, respectively. These compounds concentration-dependently inhibited NO production in intact RAW264.7 mouse macrophages stimulated by lipopolysaccharide (LPS)/interferon-γ. At 100 μM, FR038251, FR038470, FR191863 and aminoguanidine showed 81, 44, 54 and 78% inhibition of NO production, respectively. In an in-vivo experiment, FR038251, FR038470, FR191863 and aminoguanidine inhibited NO production in LPS-treated mice by 68, 40, 5 and 68% at an oral dose of 100 mg kg^{-1}. The in-vivo inhibitory activity of FR038251 was almost identical to that of aminoguanidine. In conclusion, the three FR compounds are iNOS inhibitors with novel structures and may be candidate compounds leading to discovery of more iNOS inhibitors in the future.

Introduction

Three distinct isoforms of nitric oxide synthase (NOS) have been identified and shown to generate nitric oxide (NO) in specific tissues for specific physiological roles (Moncada et al 1991). Although the level of NO that is generated by two constitutive isoforms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS), is low, it can express physiological functions through the activation of guanylate cyclase, leading to an accumulation of cGMP (Rapoport & Murad 1983). On the other hand, the inducible isoform of NOS (iNOS) is activated transcriptionally in various cells including macrophages and vascular smooth muscle cells by proinflammatory signals (Xie et al 1992; MacNaul & Hutchinson 1993). Once iNOS is expressed, the activity leads to high and cytotoxic NO levels that are necessary for an effective immune defence against invading pathogens. NO, the level of which is increased extremely, reacts towards a variety of biological substances other than guanylate cyclase in the body (Stamler et al 1992), acting as a non-specific mediator of tissue damage. iNOS activation is shown to be involved in pathogenesis of a variety of diseases including septic shock (MacMicking et al 1995), Parkinson disease (Liberatore et al 1999) and rheumatoid arthritis (Tsukahara et al 1996). Therefore, a specific iNOS inhibitor has the potential to become a powerful therapeutic drug for such diseases.

Many compounds that inhibit iNOS have been reported. Almost all the inhibitors are L-arginine analogues (Grant et al 1998) and the compounds containing a guanidine group or thiourea group in their structures (Southan et al 1995). Some L-arginine analogues show limited isoform selectivity concerning the inhibition of NOS. We have discovered three iNOS inhibitors, 5-chloro-1,3-dihydro-2H-benzimidazol-2-one (FR038251), 1,3(2H,4H)-isoquinolinedione (FR038470) and 5-chloro-2,4(1H,3H)-

Advanced Technology Platform Research Laboratory, Fujisawa Pharmaceutical Co., Ltd, 5-2-3, Tokodai, Tusukuba, Ibaraki 300-2698, Japan

Yasuhiro Kita, Masakazu Muramoto, Akihiko Fujikawa, Takao Yamazaki, Shintaro Nishimura

Molecular Biological Research Laboratory, Fujisawa Pharmaceutical Co., Ltd, 5-2-3, Tokodai, Tusukuba, Ibaraki 300-2698, Japan

Yasuhiro Kita, Yoshitada Notsu

Correspondence: Y. Kita, Advanced Technology Platform Research Laboratory, Fujisawa Pharmaceutical Co., Ltd, 5-2-3, Tokodai, Tusukuba, Ibaraki 300-2698, Japan. E-mail: yasuhiro_kita@po.fujisawa.co.jp

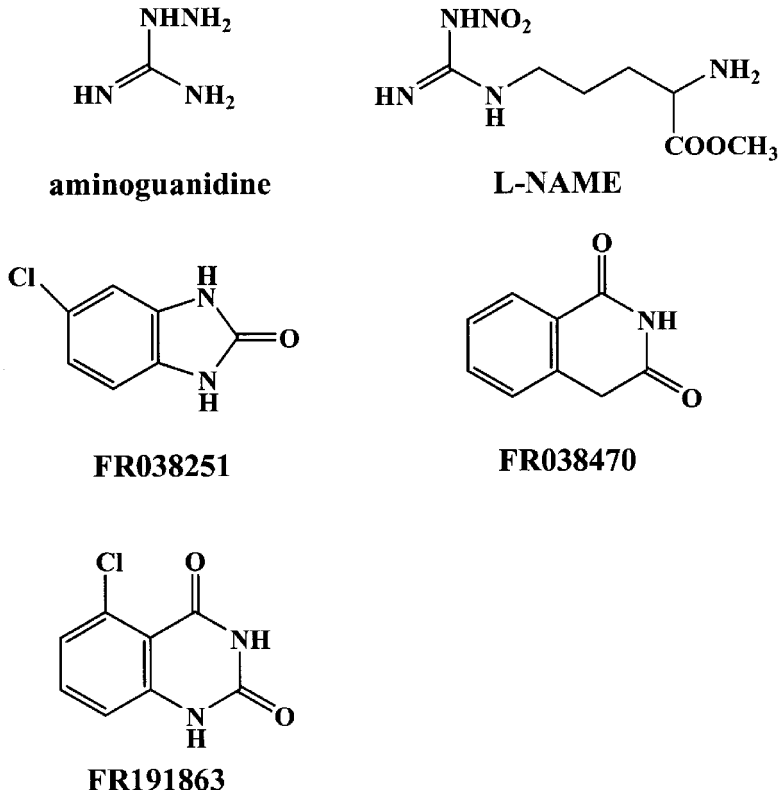

Figure 1 Chemical structures of nitric oxide synthase inhibitors.

quinazolonedione (FR191863), with novel structures (Figure 1), by random screening of a chemical library. They have two rings in their structures. Here, we report the NOS inhibitory activity of the compounds both in-vitro and in-vivo in comparison with those of aminoguanidine, a representative iNOS inhibitor. Moreover, we examined in-vivo oral activity of the compounds in order to determine their potential as therapeutic drugs for chronic diseases.

Materials and Methods

Materials

FR038251, FR038470 and FR191863 were synthesized by Fujisawa Pharmaceutical (Osaka, Japan). Aminoguanidine and L-nitro-arginine methyl ester (L-NAME) were purchased from Sigma (St Louis, MO).

Animals

Male ICR mice were supplied from Charles River Japan (Yokohama, Japan). Seven-week-old mice were used for the experiments. In an in-vivo experiment, mice were given each drug suspended with 0.5% methylcellulose at 10 mL kg^{-1}.

Enzyme preparation

iNOS or eNOS were prepared from RAW264.7 mouse macrophages and bovine pulmonary artery derived endothelium (BPAE) cells, respectively, and cultured to confluence in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS) (Sigma) in a F75-flask. nNOS was prepared from cerebella of male ICR mice. Prepared enzymes were stored at −80°C after determination of the protein concentration using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

RAW264.7 cells in 10 flasks were activated with 4 $\mu g\ mL^{-1}$ lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (Sigma) and 16 U mL^{-1} mouse recombinant interferon-γ (Genzyme, Cambridge, MA, USA) for 18 h. The cell cultures were scraped off and harvested by centrifugation after washing with PBS. The cell pellet was homogenized in 8 mL of ice-cold 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) (Nakarai, Kyoto Japan), 100 $\mu g\ mL^{-1}$ phenylmethylsufonyl fluoride (PMSF) (Sigma), 5 $\mu g\ mL^{-1}$ aprotinin (Sigma), 5 $\mu g\ mL^{-1}$ antipain (Peptide Institute, Minoh, Japan), 5 $\mu g\ mL^{-1}$ soybean trypsin inhibitor (Sigma) and 5 $\mu g\ mL^{-1}$ leupeptin (Peptide Institute), and centrifuged at 100 000 *g* for 60 min at 4°C. The supernatant was used for an iNOS enzyme assay.

BPAE cells cultured to confluence in 40 flasks were scraped off and harvested by centrifugation after washing with PBS. The cell pellet was homogenized in 28 mL

Table 1 Potency of nitric oxide synthase (NOS) inhibitors in in-vitro enzyme assays.

Compounds	IC50 (μM)		
	Inducible NOS	Neuronal NOS	Endothelial NOS
Aminoguanidine	2.1±0.5	42±5	>100 (36±2%)
L-NAME	>100 (37±2%)	2.2±0.3	1.5±0.3
FR038251	1.7±0.7	65±9	13±2
FR038470	8.8±4.5	>100 (12±5%)	25±3
FR191863	1.9±0.3	100±5	6.3±0.2

IC50 values were determined with NOS from mouse macrophage cell line RAW264.7, mouse cerebellum and bovine pulmonary artery-derived endothelium for inducible NOS, neuronal NOS and endothelial NOS, respectively. Each experiment was done in triplicate. Values are means±s.d. for three independent experiments. The numbers in parentheses show% inhibition at 100 μM.

of ice-cold 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 100 μg mL^{-1} PMSF, 5 μg mL^{-1} aprotinin, 5 μg mL^{-1} antipain, 5 μg mL^{-1} soybean trypsin inhibitor and 5 μg mL^{-1} leupeptin (buffer A), and centrifuged at 100 000 *g* for 60 min at 4°C. The pellet was re-suspended in 25 mL of ice-cold buffer A containing 10% glycerol and 1 M KCl, and centrifuged at 100 000 *g* for 60 min at 4°C. The pellet was suspended in 5 mL of ice-cold buffer A containing 10% glycerol and used for an eNOS enzyme assay.

Frozen mouse cerebella were homogenized in 10 vols (v/w) of ice-cold 20 mM Tris–HCl buffer (pH.7.5) containing 1 mM DTT, 100 μg mL^{-1} PMSF, 5 μg mL^{-1} aprotinin, 5 μg mL^{-1} antipain, 5 μg mL^{-1} soybean trypsin inhibitor and 5 μg mL^{-1} leupeptin, and centrifuged at 100 000 *g* for 60 min at 4°C. The supernatant was used for nNOS enzyme assay.

Enzyme assay

The NOS assay was performed by measuring the conversion activity of [^{3}H]arginine to [^{3}H]citrulline. Compounds were incubated in 25 mM HEPES (pH 7.5) containing each enzyme, 5 μM L-[2,3,4,5-^{3}H]arginine (80 nCi) (Amersham/Pharmacia, Cardiff, UK), 1 mM DTT, 1 mg mL^{-1} BSA, 0.75 mM $CaCl_2$, 100 μM NADPH, 10 μM Hercules and 10 μM 5,6,7,8-tetrahydobiopterin (ALEXIS, San Diego, CA, USA) in the final volume of 100 μL. Each enzyme was used at the final protein concentration of 7.5, 200 or 50 μg mL^{-1} for iNOS, nNOS, or eNOS enzyme assay, respectively. The enzyme reaction was carried out for 60 min at 37°C and terminated by adding 100 μL of 40% Dowex AG50W-X8 (Na^+ form) (200–400 mesh) (Bio-Rad) suspended with distilled water. The mixtures were immediately mixed vigorously and centrifuged for 5 min at 3000 *g*. Radioactivity of 100 μL of supernatant was determined using a liquid scintillation counter. Under these conditions, the enzyme activity was linear up to at least 120 min (data not shown). Three independent experiments were done in triplicate.

Intact cell assay

RAW264.7 mouse macrophages cultured in DMEM were harvested by centrifugation after washing by PBS and re-suspended in αMEM containing 10% FBS (without phenol red). At 3 h after transfer into a 96-well plate at the density of 5×10^4 cells/well, RAW264.7 cells were activated by 4 μg mL^{-1} LPS and 10 U mL^{-1} mouse recombinant interferon-γ for 20 h. Tested compounds were added to the 96-well plate 1 h before the activation by LPS/interferon-γ. The cell assay was performed in a volume of 100 μL. After activation for 20 h, 100 μL of a Griess reagent (Bell et al 1963) was directly added into the assay medium and the nitrite concentration was measured using a microplate reader (Bio-Rad) at the wavelength of 570 nm. Three separate experiments were done.

In-vivo assay in mice

At 5 h after the intraperitoneal injection of 0.3 mg kg^{-1} LPS or saline (7.5 mL kg^{-1}) to male ICR mice, blood was collected into the tube containing EDTA (final concentration 1 mg mL^{-1}) from the abdominal aorta under light anaesthesia. The compounds or vehicle were orally administered 30 min before the LPS injection. Plasma was obtained by the centrifugation of blood. Plasma nitrite/nitrate level was determined with a Griess reagent after the reduction of nitrate to nitrite with 0.02 U nitrate reductase (Sigma) in 125 μL of potassium phosphate buffer (pH 7.5) containing 360 μM NADPH and 10 μM FAD. Experiments were done with 6–8 mice in each group.

Statistical analysis

Data are presented as the mean±s.d. for the indicated number of experiments. The IC50 values express the drug concentration required to produce 50% inhibition of citrulline production in an enzyme assay. The IC50 value was logistically calculated. For comparison between two groups (non-treated normal group and LPS-treated control group) and multiple comparison in an in-vivo experiment, the data were analysed using Student's *t*-test and one-way

analysis of variance followed by Dunnett's test, respectively.

Results and Discussion

We have discovered three compounds, FR038251, FR038470 and FR191863, with two rings in their structures by a random screening. The in-vitro inhibitory effects of these compounds on three isoforms of NOS were compared with aminoguanidine, a typical iNOS inhibitor, and L-NAME, a nNOS and eNOS dual inhibitor (Table 1). FR038251, FR038470 and FR1919863 inhibited mouse iNOS with IC50 values of 1.7, 8.8 and 1.9 μM, respectively. Inhibitory activities of FR038251 and FR191863 were almost identical to that of aminoguanidine (IC50 = 2.1 μM). The potency of FR038470 was approximately 4-times weaker than that of other compounds. The FR compounds had iNOS selectivity 38- and 8-times, >11- and 3-times, and 53- and 3-times compared with rat nNOS and bovine eNOS, respectively; aminoguanidine had iNOS selectivity 20- and >48-times, respectively. The three FR compounds showed more potent effects against iNOS compared with constitutive NOS. In particular, FR038251 and FR191863 had superior iNOS/nNOS selectivity compared with aminoguanidine. The iNOS/eNOS selectivity of the three FR compounds was less than that of aminoguanidine. On the contrary, L-NAME had IC50 values of 2.2 and 1.5 μM on nNOS and eNOS, respectively; it inhibited iNOS by 37% at 100 μM. Thus, L-NAME showed higher selectivity to constitutive NOS compared with iNOS, but had no selectivity between nNOS and eNOS.

The three isoforms of NOS are enzymes that act intracellularly. Therefore, the NO production assay with intact cells is important in order to estimate the in-vivo activity of the NOS inhibitors. We used RAW264.7 mouse macrophages stimulated by LPS/interferon-γ as an intact cell assay. FR compounds and aminoguanidine concentration-dependently inhibited NO production in the stimulated cells for 20 h (Table 2). At 100 μM, FR038251, FR038470 and FR1919863 showed 81, 44 and 54% inhibition of NO production, respectively. Aminoguanidine inhibited NO production in RAW264.7 cells by 78% at 100 μM. The

Table 2 Inhibitory effects of nitric oxide synthase (NOS) inhibitors on nitric oxide (NO) production in intact RAW264.7 mouse macrophages stimulated by lipopolysaccharide (LPS)/interferon-γ.

Compounds	Concentration (μM)	Inhibition (%)[a]
Aminoguanidine	1	13±2
	10	32±2
	100	78±3
FR038251	1	4.0±0.9
	10	18±2
	100	81±2
FR038470	1	−4.3±1.0
	10	28±2
	100	44±2
FR191863	1	4.0±2.6
	10	19±1
	100	54±2

[a]% Inhibition of nitrite/nitrate production. NOS inhibitors were supplied 60 min before the stimulation by LPS/interferon-γ. NO production was determined as accumulated nitrite concentration in medium with a Griess reagent 20 h after the stimulation by LPS/interferon-γ. Data are means±s.d. for three experiments.

Table 3 Inhibitory effects of nitric oxide synthase (NOS) inhibitors on nitric oxide (NO) production in lipopolysaccharide (LPS)-treated mice.

Compounds	Dose (mg kg^{-1})	Nitrite/nitrate (μM)[a]	Inhibition (%)[b]	n[c]
Non-treated	–	39±13**	100	7
Control	–	190±40	0	7
Aminoguanidine	10	158±79	21	8
	32	145±40	30	7
	100	87±21**	68	7
FR038251	10	149±50	27	7
	32	165±45	17	7
	100	87±48**	68	7
FR038470	10	158±17	21	6
	32	159±44	21	6
	100	130±40*	40	7
FR191863	100	182±40	5	6

[a]Concentration of plasma nitrite/nitrate. [b]% Inhibition of plasma nitrite/nitrate production. [c]The number of animals used in each group. NO production was determined as accumulated nitrite/nitrate concentration in plasma with a Griess reagent 5 h after an intraperitoneal injection of LPS. NOS inhibitors were given orally 30 min before the administration of LPS. Data are means±s.d. *$P < 0.05$ and **$P < 0.01$, significantly different compared with LPS-treated group without NOS inhibitors (control group). Plasma nitrite/nitrate concentration in the control group was significantly higher than that in the normal group without LPS-treatment (non-treated group).

potency of FR038251 in an intact cell assay was almost identical to that of aminoguanidine. FR038251 and aminoguanidine had almost the same iNOS inhibitory potency in an in-vitro enzyme assay. These findings suggest that FR038251 and aminoguanidine have equal capacity to penetrate into cells. On the other hand, although FR191863 had almost the same potency as FR038251 and aminoguanidine in an in-vitro enzyme assay, it showed weaker inhibition of NO production in RAW264.7 cells than FR038251 and aminoguanidine. These data indicate that FR191863 is less able to access the intracellular portion. Thus, the compounds have different capacities to penetrate into cells. It is therefore important to evaluate the penetration capacity of the compounds by an NO production assay with intact cells between an in-vitro enzyme assay and an in-vivo assay.

We evaluated the in-vivo NOS inhibitory activity of the FR compounds. The tested compounds were administered orally in order to determine oral activity. iNOS activation is shown to be involved in pathogenesis of a variety of diseases. In order to treat chronic diseases such as Parkinson disease (Liberatore et al 1999) and rheumatoid arthritis (Tsukahara et al 1996), chronic drug treatment is required. Our aim was to discover an orally active iNOS inhibitor that can treat chronic diseases as well as acute diseases such as septic shock (MacMicking et al 1995). Table 3 shows the effects of the FR compounds administered orally on in-vivo NO production in LPS-stimulated mice. FR038251 and FR038470 significantly inhibited NO production by 68 and 40%, respectively, at an oral dose of 100 mg kg^{-1}, whereas FR191863 did not show inhibition at the same dose. Aminoguanidine showed significant inhibition at an oral dose of 100 mg kg^{-1} and the inhibitory activity (68% inhibition) was the same as that of FR038251. As mentioned above FR038251 and aminoguanidine showed almost the same iNOS inhibitory potency in an in-vitro enzyme assay and in an intact cell assay. These finding indicate that FR038251 and aminoguanidine have equivalent oral activity. Although we do not have data concerning the blood concentration after oral administration of the compounds, they may maintain similar blood concentrations during 5 h after the oral administration. FR038470 inhibited NO production significantly, but more weakly than FR038251. On the contrary, FR191863 did not inhibit NO production at 100 mg kg^{-1}. FR191863 may have inferior oral absorption and/or stability in blood. Thus FR compounds with similar two-ring structures have different oral activity and/or stability in blood.

In this study, we determined the in-vitro and in-vivo activities of three novel iNOS inhibitors in comparison with aminoguanidine. The compounds showed different profiles not only in the potency of iNOS inhibition, but also in enzyme selectivity, capacity to penetrate into cells, and oral activity. FR038251 had almost identical iNOS inhibitory activity to aminoguanidine both in-vitro and in-vivo. It is a new finding that such compounds with two rings in their structures have iNOS inhibitory activity and the chemical structure of these compounds may have the potential for further structural optimization leading to improved activity and selectivity for iNOS inhibition.

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