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RESEARCH PAPER

The benzoxathiolone LYR-71 down-regulates interferon-γ-inducible pro-inflammatory genes by uncoupling tyrosine phosphorylation of STAT-1 in macrophages

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Background and purpose: Benzoxathiolone derivatives have shown anti-inflammatory and immunomodulatory potential in acne and psoriatic disorders. However, little is known about the molecular basis for these pharmacological effects. In this study, we decided to investigate the anti-inflammatory actions of a benzoxathiolone derivative LYR-71, 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one, in interferon (IFN)-γ-activated macrophages.
Experimental approach: RAW 264.7 macrophages or primary macrophages, derived from bone marrow of C3H/HeJ mice, were stimulated with IFN-γ in the presence of LYR-71. Nitric oxide (NO) or chemokine production was measured by Griess reaction or enzyme-linked immunosorbent assay. RAW 264.7 cells were used to examine the molecular mechanisms of LYR-71 in modulating IFN-γ-induced inflammatory responses.
Key results: LYR-71 down-regulated IFN-γ-induced transcription of inducible NO synthase, IFN-γ-inducible protein-10 and the monokine induced by IFN-γ genes in macrophages. This effect was mediated by uncoupling tyrosine phosphorylation of the signal transducer and activator of transcription (STAT)-1 in response to IFN-γ. LYR-71 directly inhibited the *in vitro* catalytic activity of Janus kinase (JAK)-2. Further, the inhibitory actions of LYR-71 on IFN-γ-induced STAT-1 phosphorylation and NO production were consistently abolished in the presence of peroxyvanadate, implying another target dependent on protein tyrosine phosphatase.
Conclusions and implications: Taken together, LYR-71 could restrain IFN-γ-induced inflammatory responses through uncoupling the tyrosine phosphorylation of STAT-1, an activation index of JAK–STAT-1 signalling, in macrophages. These results may provide a molecular mechanism underlying anti-inflammatory actions shown by benzoxathiolone derivatives.
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Keywords: benzoxathiolone derivative; anti-inflammatory action; nitric oxide; chemokine; Janus kinase; STAT-1; IFN-γ; macrophages

Abbreviations: GAS, IFN-γ-activated sequence; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; IP-10, IFN-γ-inducible protein-10; IRF, IFN regulatory factor; ISRE, IFN-stimulated regulatory element; JAK, Janus kinase; LPS, lipopolysaccharide; LYR-71, 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one; M-CSF, macrophage-colony stimulating factor; MIG, monokine induced by IFN-γ; PTPase, protein tyrosine phosphatase; SHP, SH2 domain-containing tyrosine phosphatase; STAT, signal transducer and activator of transcription; TLR, toll-like receptor

Introduction

Interferon (IFN)-γ, a prototypical Th1 cytokine, is a major activator of macrophages for inflammatory response and cellular immunity (Stark *et al.*, 1998; Schroder *et al.*, 2004). IFN-γ exerts its biological effects through specific binding to the

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heterodimeric receptor, IFN-γ receptor-1 and IFN-γ receptor-2 (Bach *et al*., 1997). Upon ligation of IFN-γ, the IFN-γ receptors are oligomerized and the receptor-associated Janus kinase (JAK)-1 and JAK-2 are activated by trans-phosphorylation (Igarashi *et al*., 1994; Kotenko *et al*., 1995). These JAKs sequentially phosphorylate the intracellular domain of IFN-γ receptors, which serves as the docking site of the signal transducer and activator of transcription (STAT)-1 or other substrates, leading to JAKs-mediated phosphorylation of STAT-1 at Tyr-701 (Igarashi *et al*., 1994; Quelle *et al*., 1995). Tyr^{701}-phosphorylated STAT-1 is released from the receptor, forms a homodimer to function as a transcription factor and is translocated to the nucleus, where it specifically binds to the IFN-γ-activated sequence (GAS) motif for transcriptional control of target genes (Decker *et al*., 1991; Darnell *et al*., 1994). STAT-1-regulated gene transcription has been shown to mediate many, but not all, of the immune and inflammatory actions of IFN-γ (Ramana *et al*., 2002).

IFN regulatory factor (IRF)-1 is another transcription factor, rapidly inducible upon IFN-γ stimulation, and its expression is dependent on the JAK–STAT-1 signalling (Sims *et al*., 1993). Upon entry into the nucleus, IRF-1 binds to the IFN-stimulated regulatory element (ISRE), participating in the transcriptional control of IFN-γ-regulated secondary genes (Au *et al*., 1995). Thereby, IFN-γ-regulated gene transcription is mediated through the GAS and/or ISRE motifs on promoter regions of target genes, including IRF-1, inducible nitric oxide synthase (iNOS), IFN-γ-inducible protein-10 (IP-10) and the monokine induced by IFN-γ (MIG) (Lowenstein *et al*., 1993; Ohmori and Hamilton, 1993; Wong *et al*., 1994).

Tyrosine phosphorylation of STAT-1, an activation index of JAK–STAT-1 signalling in response to IFN-γ, is controlled by the balance between JAKs-mediated kinase activity and feedback mechanisms, including protein tyrosine phosphatase (PTPase) (Yamada *et al*., 2003; Valentino and Pierre, 2006). In particular, PTPases such as SH2 domain-containing tyrosine phosphatase (SHP)-1 and SHP-2 have been implicated in the regulation of IFN-γ signalling. SHP-2 functions as a negative regulator of IFN-γ-activated STAT-1 signalling and directly catalyses the dephosphorylation of Tyr^{701}-phosphorylated STAT-1 (You *et al*., 1999; Wu *et al*., 2002). A stimulatory effect of SHP-2 on JAK activity was also reported (David *et al*., 1996; You and Zhao, 1997). SHP-1 or -2 has two consecutive SH2 domains and a catalytic domain (Hof *et al*., 1998; Yang *et al*., 2003). SHPs are inactive in the resting cells, as the catalytic domain of the enzyme interacts with its N-terminal SH2 domain, restricting access of substrates (Pei *et al*., 1994). The catalytic domain of SHP-1 or -2 is activated after its docking on tyrosine-phosphorylated substrates (Jiao *et al*., 1996; You *et al*., 1999). SHPs are also activated by the phosphorylation of two-tail tyrosine residues, which serves as an adaptor function to recruit substrates or in itself stimulates PTPase activity (Vogel *et al*., 1993; Minoo *et al*., 2004).

Benzoxathiolone derivatives have been used for the treatment of acne and are reported to have antipsoriatic and antibacterial properties (Goeth and Wildfeuer, 1969; Lius and Sennerfeldt, 1979). However, little is known about the molecular basis for these pharmacological properties. This study provides a molecular mechanism underlying anti-inflammatory properties of benzoxathiolone derivatives.

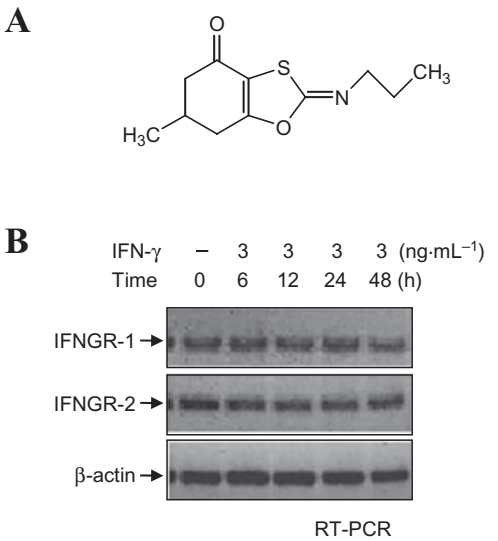

Figure 1 Chemical structure of 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (LYR-71) and cellular levels of interferon (IFN)-γ receptor-1 and -2 (IFNGR-1 and -2). (A) Chemical structure of LYR-71. (B) RAW 264.7 cells were stimulated with IFN-γ for 6–48 h. Total RNA of the cells was subjected to a reverse transcription-polymerase chain reaction (RT-PCR) analysis with β-actin gene as an internal control.

Here, we demonstrated that a benzoxathiolone derivative LYR-71, 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3] oxathiol-4-one (Figure 1A), could down-regulate the transcription of pro-inflammatory genes in IFN-γ-activated macrophages by uncoupling the tyrosine phosphorylation of STAT-1.

Methods

Animals and isolation of macrophages-derived from bone marrows

All animal care and experimental procedures were approved by the CBNU Animal Experimentation Ethics Committee. C3H/HeJ mice (lacking the toll-like receptor TLR-4; TLR-$4^{-/-}$) were obtained from Korea Research Institute of Bioscience and Biotechnology (Chungbuk, Korea) and housed in pathogen-free conditions at 21–24°C and 40–60% humidity under a 12 h light/dark cycle. Macrophages were derived from bone marrow of male mice (5–6 weeks of age). Briefly, bone marrow cells were flushed out from femurs and tibias. After lysing red blood cells, whole bone marrow cells (2×10^6 cells·mL^{-1}) were cultured in complete media containing macrophage-colony stimulating factor (M-CSF, 10 ng·mL^{-1}). On culture day 3, the media were replaced with fresh complete media containing M-CSF (10 ng·mL^{-1}), and on day 6, half of the media was changed in the presence of M-CSF (10 ng·mL^{-1}). On day 8, adherent cells were harvested and then used as primary macrophages.

Cell culture

RAW 264.7 macrophages were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were

cultured in Dulbecco's modified Eagle's media supplemented with heat-inactivated 10% fetal bovine serum (FBS), benzylpenicillin potassium (143 U·mL^{-1}) and streptomycin sulphate (100 μg·mL^{-1}) under 37°C and 5% CO_2 atmosphere.

Reverse transcription-polymerase chain reaction (RT-PCR)
The cells were pretreated with LYR-71 for 2 h, and stimulated with IFN-γ (3 ng·mL^{-1}) for 4–6 h, in the presence of LYR-71. Total RNA of the cells was subjected to semiquantitative RT-PCR, using an RNA PCR kit (Bioneer, Daejeon, Korea). Briefly, total RNA was reversely transcribed at 42°C for 1 h, and then subjected to 25–30 cycles of PCR consisting of 30 s denaturation at 94°C, 30 s annealing at 50–60°C and 90 s extension at 72°C. The sequences of primers for RT-PCR and the sizes of PCR products are as follows: IFN-γ receptor-1 [455 base pair (bp)], sense 5′-GACGTCTGTATCCCTCCTTT CCATG-3′ and antisense 5′-CTACAAGGGCCTGACTGGTT ACTAC-3′; IFN-γ receptor-2 (535 bp), sense 5′-GTCATACACT TCTCCCCTCCCTTTG-3′ and antisense 5′-GCACATCATCT CGCTCCTTTTCTGG-3′; iNOS (457 bp), sense 5′-GTCAAC TGCAAGAGAACGGAGAAC-3′ and antisense 5′-GAGCTCC TCCAGAGGGTAGGCT-3′; IP-10 (430 bp), sense 5′-ACTC ACTCAGTTTGTTGAGTCATTC-3′ and antisense 5′-TTTG ATTAGTACTGTAGGGTTAATG-3′; MIG (421 bp), sense 5′-CTT CAGCCCCAGCAGTGTATTCTTT-3′ and antisense 5′-AGA GAACCTGGGAGTAGACAAGGTA-3′; IRF-1 (470 bp), sense 5′-ACATAACTCCAGCACTGTCACCGTG-3′ and antisense 5′-GGTCAGAGACCCAAACTATGGTGCA-3′; and β-actin (745 bp), sense 5′-CACCACACCTTCTACAATGAGCTGC-3′ and antisense 5′-GCTCAGGAGGAGCAATGATCTTGAT-3′. RT-PCR products were finally resolved on agarose gels by electrophoresis and stained with ethidium bromide.

Griess reaction for nitric oxide (NO) quantification
The cells were stimulated with IFN-γ (3 ng·mL^{-1}) for 24 h, in the presence of LYR-71. Aliquots of the culture media were reacted with the same volume of 0.05% sulfanilamide and 0.05% *N*-(1-naphthyl)ethylenediamine, and then absorbance values at 540 nm were measured, using sodium nitrite as the standard.

Cell proliferation assay
The cells were incubated with various concentrations of LYR-71 for 24 h, in the presence of IFN-γ. They were then exposed to a water-soluble form of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (Dojindo Lab., Kumamoto, Japan) for 3 h, and absorbance values measured at 450 nm.

Enzyme-linked immunosorbent assay (ELISA)
The cells were stimulated with IFN-γ (3 ng·mL^{-1}) for 24 h, in the presence of LYR-71. Amounts of IP-10 and MIG in the culture media were measured using ELISA kits (R&D Systems, Minneapolis,
MN, USA).

Immunoblotting analysis
The cells were pretreated with LYR-71 for 2 h and stimulated with IFN-γ (3 ng·mL^{-1}) for the indicated times, in the presence of LYR-71. Cell extracts were resolved on sodium dodecyl sulphate (SDS)-acrylamide gels by electrophoresis and then transferred to a polyvinylidene difluoride membrane. Either 5% nonfat milk in phosphate-buffered saline containing Tween 20 or 5% bovine serum albumin in Tris-buffered saline containing Tween 20 was used as the blocking buffer. The blots were usually incubated at 4°C overnight with primary antisera, and then incubated at room temperature for 2–5 h with horseradish peroxidase-labelled secondary antisera. Immune complexes on the blots were finally visualized by exposure to X-ray film after reacting with an enhanced chemiluminescence reagent (GE Healthcare, Chalfont, UK).

DNA transfection and luciferase gene reporter assay
The cells were transiently transfected with each luciferase reporter construct of piNOS-Luc, pIRF-1-Luc, pGAS-Luc or pISRE-Luc, using lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Transfection reactions were pooled and then redistributed at 5 × 10^5 cells per well. The transfected cells were stimulated with IFN-γ (3 ng·mL^{-1}) for 16 h, in the presence of LYR-71. Cell extracts were subjected to dual-luciferase reporter assay (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to *Renilla* activity to account for transfection efficiency. Protein levels were measured using a dye-based assay kit (Bio-Rad, Hercules, CA, USA).

Nuclear protein extraction and electrophoretic mobility shift assay
The cells were pretreated with LYR-71 for 2 h and stimulated with IFN-γ (3 ng·mL^{-1}) for 40 min. The cells were then disrupted in a lysis buffer (10 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet P-40, 0.1 mM EDTA, 1 mM DTT and 0.5 mM PMSF) on ice for 20 min. After centrifugation, pellets were resuspended in an extraction buffer (20 mM HEPES, pH 7.9, 50 mM $MgCl_2$, 420 mM KCl, 20% glycerol, 0.1 mM EDTA, 1 mM DTT and 0.5 mM PMSF) and incubated on ice for 30 min. After centrifugation, supernatants were used as the nuclear extracts. A double-stranded oligonucleotide of 5′-GCCTGATTTCACCGAAATGACGGC-3′ was used as the probe. The ^{32}P end-labelled oligonucleotide was mixed with nuclear extracts in a binding buffer [10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 50 μg·mL^{-1} poly(dI-dC) and 4% glycerol] and incubated on ice for 30 min. The reaction mixtures were resolved on non-denaturing acrylamide gels by electrophoresis and then subjected to autoradiography.

Immunoprecipitation
The cells were pretreated with LYR-71 for 2 h and stimulated with IFN-γ (3 ng·mL^{-1}) for 10 min. Cell extracts (500 μg) were incubated with anti-JAK-1 or anti-JK-2 antibody (1 μg each) at 4°C overnight, and precipitated with protein G-agarose beads (GE Healthcare) for 2 h.

In vitro *assay of JAK autophosphorylation*
The immunoprecipitates with anti-JAK-1 or anti-JK-2 antibody were washed with a kinase buffer (10 mM HEPES, pH

7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 50 mM NaF, and 0.1 mM Na_3VO_4). The immunoprecipitates were resuspended in the kinase buffer containing LYR-71 and 5 μCi [γ-^{32}P]adenosine triphosphate (GE Healthcare), and incubated at 30°C for 30 min with intermittent agitation. The reaction mixtures were boiled in SDS-sample buffer, resolved on SDS-acrylamide gels by electrophoresis, and then subjected to autoradiography.

PTPase activity assay

The cells (1×10^6) were treated with LYR-71 in the absence or presence of peroxyvanadate for 2 h, and then disrupted in a lysis buffer (50 mM Tris, pH 7.0, 1% Nonidet P-40, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 25 μg·mL^{-1} aprotinin, 25 μg·mL^{-1} leupeptin). PTPase activities in the cell lysates were determined as described previously with minor modifications (Kozlowski *et al.*, 1993). Briefly, the substrate *p*-nitrophenyl phosphate (2 mM) was mixed with an assay buffer (50 mM HEPES, pH 7.5, 0.1% mercaptoethanol) and then cell lysates were added. Initial dephosphorylation rate of *p*-nitrophenyl phosphate was measured as the increase of absorbance at 410 nm per min.

Statistical analysis

Data are expressed as means ± standard deviation, and were subjected to ANOVA followed by Dunnet's test. Statistical differences were considered significant when $P < 0.05$.

Materials

LYR-71 (>97% purity) was prepared as described previously (Kim *et al.*, 2008c). FBS and other culture materials were purchased from Invitrogen. Antibodies against iNOS, JAK-1 or glyceraldehyde 3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); those against IRF-1, STAT-1, JAK-2 or phosphotyrosine (4G10) from Upstate Technology (Charlottesville, VA, USA); and antibody against Tyr^{701}-phosphorylated (pY)-STAT-1 from Cell Signaling Technology (Danvers, MA, USA). STAT-1- or IRF-1-dependent reporter plasmids of pGAS-Luc and pISRE-Luc were purchased from BD Biosciences Clontech (Palo Alto, CA, USA). Promoter-dependent reporter constructs of piNOS-Luc and pIRF-1-Luc have been described previously (Lowenstein *et al.*, 1993; Ohmori *et al.*, 1997). All other chemicals including IFN-γ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard pharmacological nomenclature was used according to Alexander *et al.* (2008).

Results

In the present study, we investigated the effects of LYR-71 (Figure 1A) on JAK-STAT-1-regulated expression of inflammatory genes in IFN-γ-activated macrophages, and also provided a molecular basis of the action. As model macrophages, RAW 264.7 cells and primary macrophages-derived from bone marrows of C3H/HeJ (TLR-4$^{-/-}$) mice were used. IFN-γ receptors-1 and -2 were first identified in RAW 264.7 cells using an RT-PCR analysis, and their cellular levels were unchanged, even in the activated conditions after exposure to IFN-γ (Figure 1B).

LYR-71 efficiently inhibits IFN-γ-induced production of NO or chemokine in macrophages

To investigate anti-inflammatory activity of LYR-71, we first quantified NO in RAW 264.7 cells. Amounts of nitrite, a stable metabolite of NO, were quite low with 5–7 μM in the resting cells, but markedly increased up to 39–42 μM, upon exposure to IFN-γ alone (Figure 2A). Treatment with LYR-71 inhibited IFN-γ-induced nitrite production in a concentration-dependent manner, showing an IC_{50} value of 2.4 μM (Figure 2A). Previous studies have shown that LYR-71 and its analogue can uncouple the NF-κB activating pathway, resulting in the inhibition of TLR-4-mediated production of inflammatory mediators such as NO, interleukin (IL)-1β, IL-6 and

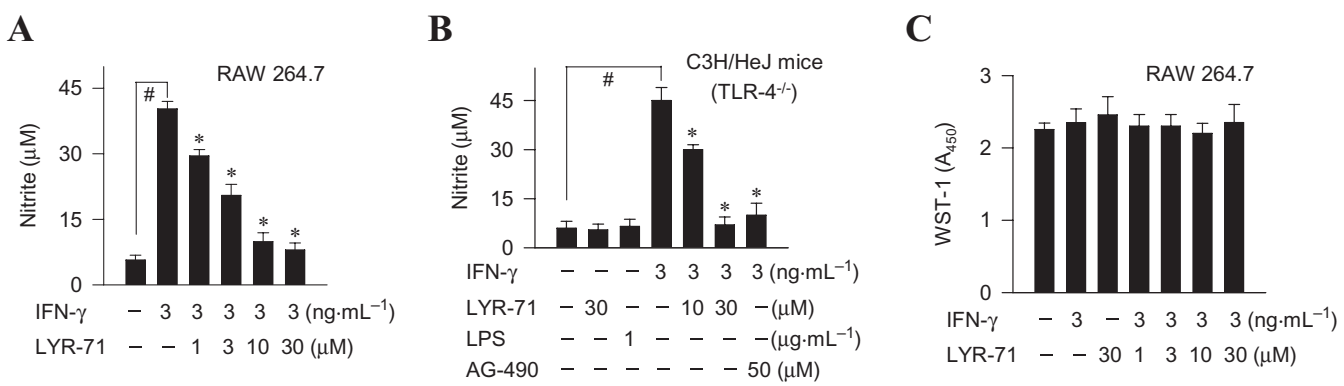

Figure 2 Interferon (IFN)-γ-induced nitric oxide (NO) production. RAW 264.7 cells were stimulated with IFN-γ for 24 h, in the presence of 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (LYR-71) (A). Primary macrophages-derived from bone marrow of C3H/HeJ (TLR-4$^{-/-}$) mice were stimulated with IFN-γ or lipopolysaccharide (LPS) for 24 h, in the presence of LYR-71 or AG-490 (B). Amounts of nitrite, a stable metabolite of NO, were determined using sodium nitrite as a standard. Data are means ± standard deviation (SD) from three to five separate experiments. $^{\#}P < 0.05$ versus media alone-added group. $^{*}P < 0.05$ versus IFN-γ alone-stimulated group. (C) RAW 264.7 cells were incubated with LYR-71 plus IFN-γ for 24 h, and then absorbance values at 450 nm (A_{450}) measured after exposure to 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-1) solution. Data are means ± SD from three separate experiments.

tumour necrosis factor-α in lipopolysaccharide (LPS)-activated macrophages (Kim *et al*., 2008a,c). To exclude LPS signalling, primary macrophages-derived from bone marrows of C3H/HeJ (TLR-4$^{-/-}$) mice were then used. The primary cells also increased NO production on treatment with IFN-γ, but not by LPS or LYR-71 alone (Figure 2B). This NO production by IFN-γ was also inhibited in the presence of LYR-71 (Figure 2B). To determine whether the activity of JAKs, tyrosine kinases that phosphorylate STAT-1, is required for NO production in response to IFN-γ, we treated the primary macrophages with a specific JAK-2 inhibitor AG-490 (Meydan *et al*., 1996) before stimulation with IFN-γ. As shown in Figure 2B, treatment with AG-490 completely prevented NO production in response to IFN-γ.

We next examined whether LYR-71 could affect IFN-γ-induced production of chemokines. Upon exposure to IFN-γ alone, RAW 264.7 cells released increased amounts of IP-10, more than 10-fold higher than the basal levels (Table 1). Treatment with LYR-71 inhibited IFN-γ-induced IP-10 production in a concentration-dependent manner, showing an IC_{50} value of 3.5 μM (Table 1). In a parallel experiment, LYR-71 also inhibited IFN-γ-induced MIG production with an IC_{50} value of 2.6 μM (Table 1). However, LYR-71 at the effective concentrations for anti-inflammatory responses did not affect the proliferation of RAW 264.7 cells (Figure 2C), thus excluding a nonspecific cytotoxicity as a possible explanation for the decreased cytokine output.

Table 1 IFN-γ-induced IP-10 or MIG production

Sample	*IP-10 (ng·mL^{-1})*	*MIG (pg·mL^{-1})*
Media alone	1.2 ± 0.4	8.8 ± 3.2
LYR-71 (30 μM) alone	2.0 ± 0.6	8.0 ± 0.9
IFN-γ alone	15.9 ± 1.1$^{\#}$	222.7 ± 23.5$^{\#}$
IFN-γ + LYR-71 (1 μM)	12.9 ± 1.5	162.6 ± 9.2*
IFN-γ + LYR-71 (3 μM)	8.4 ± 0.8*	74.3 ± 17.4*
IFN-γ + LYR-71 (10 μM)	3.9 ± 1.2*	30.1 ± 5.4*
IFN-γ + LYR-71 (30 μM)	3.1 ± 0.4*	9.6 ± 2.8*

$^{\#}P < 0.05$ versus media alone-added group. $^{*}P < 0.05$ versus IFN-γ alone-stimulated group.RAW 264.7 cells were stimulated with IFN-γ (3 ng·mL^{-1}) for 24 h, in the presence of LYR-71. Amounts of IP-10 or MIG in the culture media were determined by enzyme-linked immunosorbent assay. Data are means ± standard deviation from three to five separate experiments.
IFN, interferon; IP-10, IFN-γ-inducible protein-10; MIG, monokine induced by IFN-γ; LYR-71, 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one.

LYR-71 down-regulates IFN-γ-induced expression of iNOS and chemokine genes

To understand whether the effect of LYR-71 on NO production was associated with changes in protein levels of iNOS, RAW 264.7 cells were stimulated with IFN-γ in the presence of LYR-71 and then assessed by Western blot analysis. Protein levels of iNOS were quite low in the resting cells, but markedly increased upon exposure to IFN-γ alone (Figure 3A). Treatment with LYR-71 concentration-dependently decreased IFN-γ-induced protein levels of iNOS (Figure 3A). Moreover, treatment of AG-490 also inhibited the iNOS induction in response to IFN-γ (Figure 3A). We next performed an RT-PCR analysis to investigate the mRNA levels of iNOS. The iNOS mRNA was hardly detectable in RAW 264.7 cells at resting state, and was induced markedly upon exposure to IFN-γ alone (Figure 3B). This cellular induction of iNOS mRNA by IFN-γ was suppressed in the presence of LYR-71 (Figure 3B). In a parallel experiment, treatment with LYR-71 attenuated IFN-γ-induced mRNA levels of chemokine IP-10 or MIG (Figure 3B).

To delineate whether this action of LYR-71 was taking place at the transcription level, we transfected RAW 264.7 cells with piNOS-Luc construct, encoding the iNOS promoter (–1592/+183) that was fused to the luciferase gene as a reporter (Lowenstein *et al*., 1993). Upon exposure to IFN-γ alone, the transfected cells increased luciferase expression up to about 12-fold over the basal levels (Figure 3C). This luciferase induc-

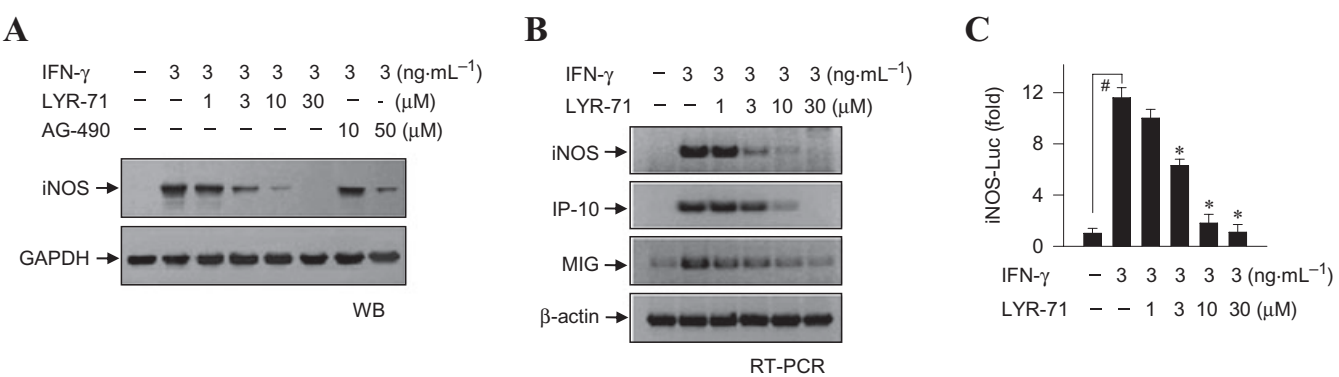

Figure 3 Interferon (IFN)-γ-induced inducible nitric oxide synthase (iNOS) or chemokine expression. (A) RAW 264.7 cells were stimulated with IFN-γ for 24 h, in the presence of 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (LYR-71) or AG-490. Cell extracts were subjected to Western blot (WB) analysis with anti-iNOS or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody. A typical blot is shown. (B) The cells were pretreated with LYR-71 for 2 h and then stimulated with IFN-γ for 4–6 h, in the presence of LYR-71. Total RNA of the cells was subjected to a reverse transcription-polymerase chain reaction (RT-PCR) analysis with β-actin gene as an internal control. A typical result is shown. (C) The cells were transfected with piNOS-Luc reporter construct, and then simulated with IFN-γ for 16 h, in the presence of LYR-71. Cell extracts were subjected to dual-luciferase assay. Luciferase expression is represented as a relative fold, in which firefly luciferase activity was normalized to the *Renilla* activity. Data are means ± standard deviation from three separate experiments. $^{\#}P < 0.05$ versus media alone-added group. $^{*}P < 0.05$ versus IFN-γ alone-stimulated group.

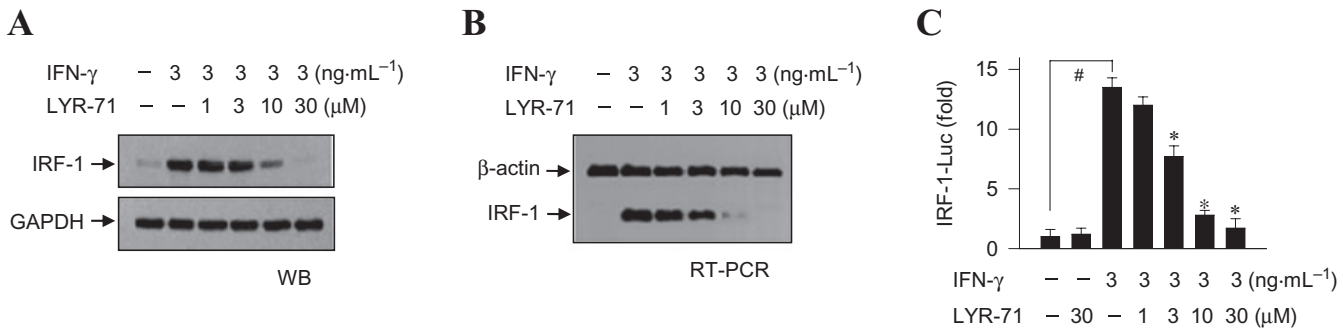

Figure 4 Interferon (IFN)-γ-induced IFN regulatory factor (IRF)-1 expression. RAW 264.7 cells were pretreated with 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (LYR-71) for 2 h and stimulated with IFN-γ for 6 h (A) or 1 h (B), in the presence of LYR-71. (A) Cell extracts were subjected to Western blot (WB) analysis with anti-IRF-1 or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody. A typical blot is shown. (B) Total RNA of the cells was subjected to a reverse transcription-polymerase chain reaction (RT-PCR) analysis with β-actin gene as an internal control. A typical result is shown. (C) The cells were transfected with pIRF-1-Luc reporter construct, and then simulated with IFN-γ for 16 h, in the presence of LYR-71. Cell extracts were subjected to dual-luciferase assay. Luciferase expression is represented as a relative fold, in which firefly luciferase activity was normalized to the *Renilla* activity. Data are means ± standard deviation from three separate experiments. $^{\#}P < 0.05$ versus media alone-added group. $^{*}P < 0.05$ versus IFN-γ alone-stimulated group.

tion in response to IFN-γ was inhibited by treatment with LYR-71 concentration-dependently with an IC_{50} value of 2.9 μM (Figure 3C).

LYR-71 suppresses IFN-γ-induced IRF-1 expression

IRF-1 is another transcription factor, inducible in response to IFN-γ, in which STAT-responsive GAS motifs appear to be essential for maximal IRF-1 transcription (Sims *et al*., 1993). To investigate the contribution of JAK-STAT-1-dependent action of LYR-71, we examined IFN-γ-induced IRF-1 expression in RAW 264.7 cells. Upon exposure to IFN-γ alone, protein levels of IRF-1 were markedly induced, which was decreased by treatment with LYR-71 (Figure 4A). Further, mRNA levels of IRF-1 in response to IFN-γ were also attenuated by treatment with LYR-71 (Figure 4B). To confirm that LYR-71's action on IFN-γ-induced IRF-1 expression occurred at the transcription level, we transfected RAW 264.7 cells with the pIRF-1-Luc construct, encoding the IRF-1 promoter (about –1.3 kilo bp) fused to the luciferase gene as a reporter (Ohmori *et al*., 1997). Upon exposure to IFN-γ alone, the transfected cells had about 14-fold increased luciferase expression over the basal levels (Figure 4C). This IFN-γ-induced luciferase expression was suppressed by treatment with LYR-71 in a concentration-dependent manner (Figure 4C).

LYR-71 inhibits IFN-γ-induced transcriptional activity of STAT-1 or IRF-1

To determine the mechanism underlying the down-regulation by LYR-71 of IFN-γ-induced transcription of IRF-1 or other pro-inflammatory genes, we first investigated whether LYR-71 could affect STAT-1 or IRF-1 transcriptional activity in macrophages. RAW 264.7 cells were transfected with pGAS-Luc or pISRE-Luc construct, containing two copies of STAT-1-responsive GAS motif or IRF-1-reponsive ISRE motif that are respectively fused to the luciferase gene as a reporter. Upon exposure to IFN-γ alone, we observed profound increases of luciferase expression in the cells harbouring pGAS-Luc (Figure 5A) or pISRE-Luc construct (Figure 5B) over

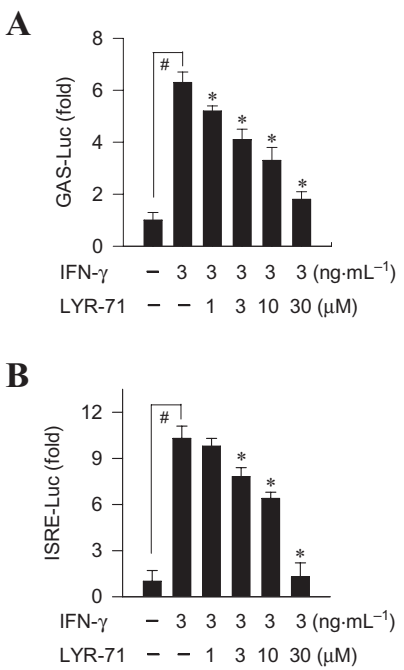

Figure 5 Interferon (IFN)-γ-induced transcriptional activity of signal transducer and activator of transcription-1 or IFN regulatory factor-1. RAW 264.7 cells were transfected with pGAS-Luc (A) or pISRE-Luc reporter construct (B). Each of the transfected cells was simulated with IFN-γ for 16 h, in the presence of 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (LYR)-71. Cell extracts were subjected to dual-luciferase assay. Luciferase expression is represented as relative-fold increase, in which the firefly luciferase activity was normalized to the *Renilla* activity. Data are means ± standard deviation from three to five separate experiments. $^{\#}P < 0.05$ versus media alone-added group. $^{*}P < 0.05$ versus IFN-γ alone-stimulated group.

the basal levels, indicating that the cellular STAT-1 and IRF-1 were functional. Treatment with LYR-71 concentration-dependently inhibited IFN-γ-induced transcriptional activity of STAT-1 with an IC_{50} value of 6.7 μM (Figure 5A). Moreover, LYR-71 inhibited transcriptional activity of IRF-1 with an IC_{50} value of 9.2 μM in the IFN-γ-activated cells (Figure 5B).

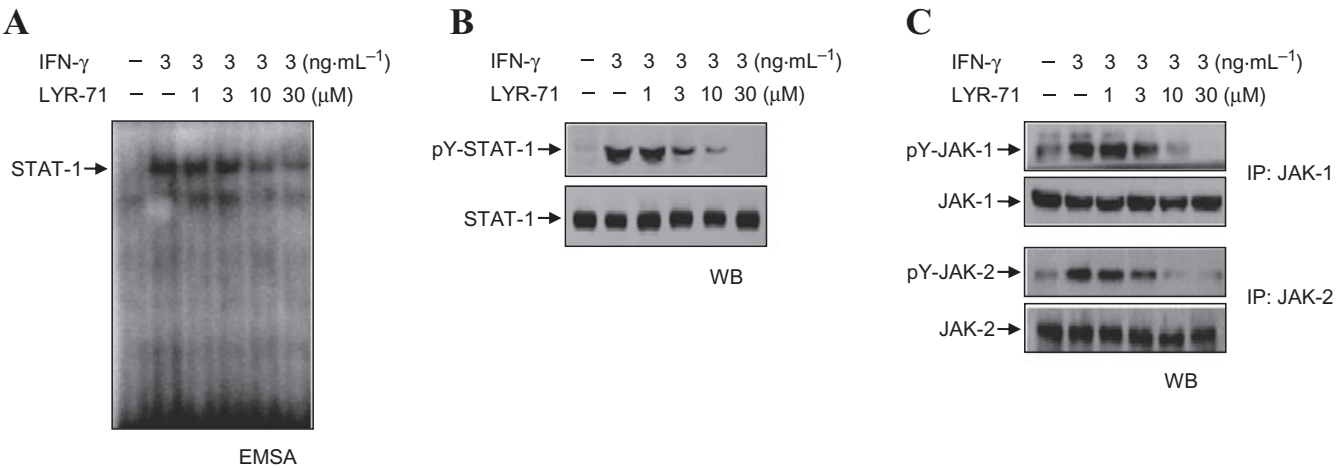

Figure 6 Interferon (IFN)-γ-induced activation of Janus kinase (JAK)–signal transducer and activator of transcription(STAT)-1 signalling. (A) RAW 264.7 cells were pretreated with 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (LYR)-71 for 2 h and stimulated with IFN-γ for 40 min, in the presence of LYR-71. Nuclear extracts were mixed with a ^{32}P end-labelled oligonucleotide containing STAT-1-responsive IFN-γ-activated sequence (GAS) motif. The reaction mixtures were resolved on nondenaturing acrylamide gels by electrophoresis, and then subjected to autoradiography for electrophoretic mobility shift assay (EMSA). The cells were pretreated with LYR-71 for 2 h and stimulated with IFN-γ for 15 min (B) or 10 min (C). (B) Cell extracts were subjected to Western blot (WB) analysis with anti-pY-STAT-1 (Tyr-701) or anti-STAT-1 antibody. (C) Cell extracts were immunoprecipitated with anti-JAK-1 or anti-JAK-2 antibody. Each immunoprecipitate (IP) was subjected to WB analysis with anti-phosphotyrosine antibody 4G10, and anti-JAK-1 or anti-JAK-2 antibody.

LYR-71 inhibits IFN-γ-induced STAT-1 binding to the GAS motif

STAT-1 transcriptional activity in IFN-γ-activated macrophages is preceded by the nuclear import and binding to DNA of the transcription factor (Schroder *et al.*, 2004; Takaoka and Yanai, 2006). We determined whether LYR-71 could affect the DNA binding ability of STAT-1 in response to IFN-γ. RAW 264.7 cells were stimulated with IFN-γ in the presence of LYR-71. Nuclear extracts of the cells were reacted with a GAS-specific ^{32}P end-labelled oligonucleotide and then subjected to an electrophoretic mobility shift assay. Upon exposure to IFN-γ alone, STAT-1 binding to the GAS-specific oligonucleotide was significantly increased over the basal levels (Figure 6A). Preliminary experiments had shown a specificity of the STAT-1 binding to the GAS motif through complete displacement in the presence of a 50-fold excess of cold GAS-specific oligonucleotide and also supershifting by an anti-STAT-1 antibody (data not shown). Treatment with LYR-71 concentration-dependently inhibited the DNA binding ability of STAT-1 in response to IFN-γ (Figure 6A).

LYR-71 inhibits IFN-γ-induced tyrosine phosphorylation of JAK and STAT-1 proximal to the receptor

The DNA binding ability and transcriptional activity of STAT-1 in response to IFN-γ are dependent on early activation events such as the trans-phosphorylation of JAK-1 and -2, and then JAKs-mediated phosphorylation of STAT-1 at Tyr-701 (Bach *et al.*, 1997; Schroder *et al.*, 2004). To investigate a possible primary event affected by LYR-71, RAW 264.7 cells were stimulated with IFN-γ in the presence of LYR-71. Cell extracts were assessed by Western blot analysis using a specific antibody against Tyr^{701}-phosphorylated STAT-1. The tyrosine phosphorylation as an activation index of STAT-1 signalling was hardly detectable in the resting cells, and was markedly increased upon exposure to IFN-γ alone (Figure 6B). Treatment with LYR-71 concentration-dependently attenuated IFN-γ-increased levels of Tyr^{701}-phosphorylated STAT-1 (Figure 6B). As expected, cellular levels of total STAT-1 proteins were not significantly affected by treatment of IFN-γ and LYR-71 (Figure 6B).

We next examined whether LYR-71's action on STAT-1 phosphorylation was due to its early effect on IFN-γ-induced trans-phosphorylation of JAKs. RAW 264.7 cells were stimulated with IFN-γ in the presence of LYR-71. Cell extracts were immunoprecipitated with anti-JAK-1 or anti-JAK-2 antibody, followed by Western blot analysis with an anti-phosphotyrosine antibody (4G10). Both JAK-1 and JAK-2 were weakly phosphorylated in the resting cells, and their phosphotyrosine levels were significantly increased upon exposure to IFN-γ alone (Figure 6C). Treatment with LYR-71 concentration-dependently reduced IFN-γ-induced tyrosine phosphorylation of JAK-1 and also that of JAK-2 in the cells (Figure 6C).

Action of LYR-71 is dependent on JAK-2 and PTPase

To identify a molecular target involved in the action of LYR-71 on IFN-γ-induced tyrosine phosphorylation of JAKs and STAT-1, we first considered whether LYR-71 could directly inhibit the catalytic activity of JAK-1 or -2. RAW 264.7 cells were stimulated with IFN-γ alone for 10 min. Cell extracts were immunoprecipitated with anti-JAK-1 or anti-JAK-2 antibody, and then assessed for their catalytic activities in the presence of LYR-71. The immunoprecipitated JAK-1 or JAK-2 still exhibited catalytic activity of a ^{32}P-incorporating autophosphorylation (Figure 7A). Treatment with LYR-71 of the cell-free enzymes inhibited *in vitro* autophosphorylation of JAK-2, but not that of JAK-1 (Figure 7A).

The tyrosine phosphorylation of STAT-1 is also balanced by negative mechanisms, including PTPases (Yamada *et al.*, 2003;

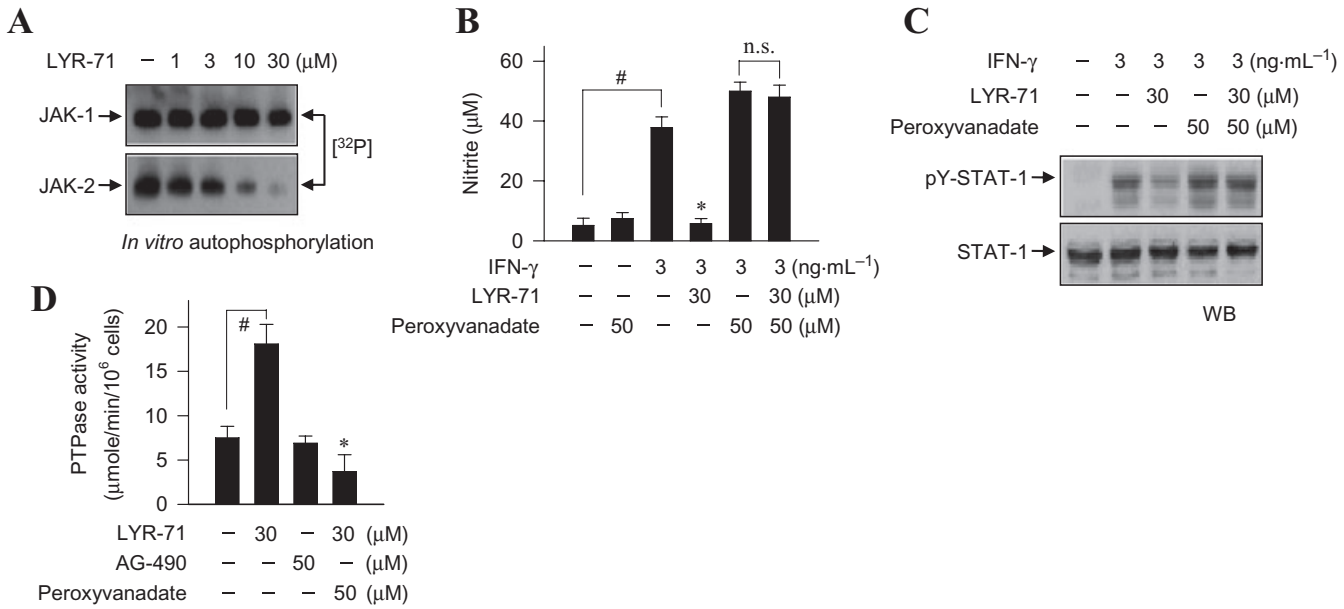

Figure 7 *In vitro* autophosphorylation of Janus kinases (JAKs) and interferon (IFN)-γ-induced nitric oxide production and signal transducer and activator of transcription (STAT)-1 phosphorylation. (A) RAW 264.7 cells were stimulated with IFN-γ (3 ng·mL^{-1}) alone for 10 min. Cell extracts were immunoprecipitated with anti-JAK-1 or anti-JAK-2 antibody. Each immunoprecipitate was treated with 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (LYR)-71 for 20 min before adding [γ-^{32}P]adenosine trisphosphate, and then *in vitro* autophosphorylation reaction was carried out for 30 min with intermittent agitation. The reaction mixtures were resolved on sodium dodecyl sulphate-acrylamide gels by electrophoresis, and then subjected to autoradiography. (B) The cells were stimulated with IFN-γ for 24 h, in the presence of LYR-71 and/or peroxyvanadate. Amounts of nitrite in the culture media were determined, using sodium nitrite as a standard. Data are means ± standard deviation (SD) from five separate experiments. $^{\#}P < 0.05$ versus media alone-added group. $^{*}P < 0.05$ versus IFN-γ alone-stimulated group. No significant difference (n.s.) between paired groups. (C) The cells were pretreated with LYR-71 for 2 h, in the absence or presence of peroxyvanadate, and then stimulated with IFN-γ for 15 min. Cell extracts were subjected to Western blot (WB) analysis with anti-pY-STAT-1 (Tyr-701) or anti-STAT-1 antibody. (D) The cells were treated with LYR-71 in the absence or presence of peroxyvanadate for 2 h. Cell lysates were tested for protein tyrosine phosphatase (PTPase) activity, assayed as the dephosphorylation rate of *p*-nitrophenyl phosphate as a substrate. Data are means ± SD from three separate experiments. $^{\#}P < 0.05$ versus media alone-added group. $^{*}P < 0.05$ versus LYR-71 alone-treated group.

Valentino and Pierre, 2006). To explore the possibility that PTPase is another regulatory target of LYR-71, we examined the effect of peroxyvanadate as a PTPase inhibitor. As reported in macrophages (Diaz-Guerra *et al.*, 1999; Chen *et al.*, 2003), peroxyvanadate alone did not increase NO production, but it significantly potentiated NO production in response to IFN-γ (Figure 7B). Under this condition, the action of LYR-71 on IFN-γ-induced NO production was interestingly abolished in the presence of peroxyvanadate (Figure 7B). To clarify whether the pharmacological action on NO production was due to modulating the tyrosine phosphorylation of STAT-1, RAW 264.7 cells were pretreated with LYR-71 in the absence or presence of peroxyvanadate and then stimulated with IFN-γ. Cell extracts were assessed by Western blot analysis with anti-Tyr^{701}-phosphorylated STAT-1 antibody. As shown in Figure 7C, peroxyvanadate slightly potentiated the cellular levels of Tyr^{701}-phosphorylated STAT-1 in response to IFN-γ, and consistently prevented the inhibitory action of LYR-71 on IFN-γ-induced tyrosine phosphorylation of STAT-1. We next confirmed whether LYR-71 could directly affect PTPase activity in the cells. Total cellular PTPase activity was significantly increased upon exposure to LYR-71 alone, but not by treatment with AG-490 alone (Figure 7D). LYR-71-increased PTPase activity and basal activity in the cells were also inhibited in the presence of peroxyvanadate (Figure 7D).

Discussion and conclusion

LYR-71 (Figure 1A) is a derivative designed from the benzofuran moiety on α-viniferin with a chemical structure of trimeric resveratrol. We previously isolated α-viniferin from *Carex humilis*, a plant of Cyperaceae family (Lee *et al.*, 1998), and reported its anti-inflammatory potential (Lee *et al.*, 1998; Chung *et al.*, 2003). However, covalent carbon–carbon or carbon–oxygen linkages in the herbal α-viniferin are recalcitrant to hydrolytic cleavage, generating monomeric structure, in mammalian cells (data not shown). We previously reported that LYR-71 could inhibit NF-κB activation, resulting in down-regulation of IL-1β expression in LPS-activated macrophages (Kim *et al.*, 2008c) and also could inhibit tumour migration/invasion in regulated upon activation, normal T-cell expressed and secreted chemokine-activated breast cancer cells by blocking STAT-3-mediated matrix metalloproteinase 9 expression (Kim *et al.*, 2008b).

Gene-knockout studies of STAT-1 provide some indication of the physiological importance of JAK–STAT signalling in the immune and inflammatory responses of IFN-γ (Meraz *et al.*, 1996; Ramana *et al.*, 2002). However, dysregulated overproduction and hyper-responsiveness to IFN-γ are implicated in the deleterious effects, including autoimmunity and tissue damage secondary to excessive inflammation (Dolhain *et al.*,

1996; von Herrath and Oldstone, 1997; Gottenberg and Chiocchia, 2007; Chen and Liu, 2009). Much attention has been focused on the pharmacological mechanisms that restrain or attenuate IFN-γ action, thus protecting the host from inflammatory disorders. The purpose of this study was to document anti-inflammatory activity of LYR-71 in IFN-γ-activated macrophages, and also to delineate a molecular basis of its action.

We first quantified inflammatory mediators in IFN-γ-activated macrophages. Treatment with LYR-71 concentration-dependently inhibited IFN-γ-induced production of NO, IP-10 or MIG in RAW 264.7 cells (Figure 2A and Table 1). Moreover, treatment of LYR-71 inhibited IFN-γ-induced NO production in primary macrophages-derived from bone marrows of C3H/HeJ (TLR-4$^{-/-}$) mice (Figure 2B), in which LPS signalling is excluded. LYR-71 also diminished IFN-γ-induced protein levels of iNOS in RAW 264.7 cells (Figure 3A), differentially attenuated IFN-γ-induced mRNA levels of iNOS, IP-10 or MIG (Figure 3B) as well as inhibited IFN-γ-induced promoter activity of iNOS gene (Figure 3C). These results indicate that LYR-71 could restrain inflammatory responses in IFN-γ-activated macrophages through down-regulating the expression of inflammatory genes at the transcription level.

Another transcription factor of IRF-1 is rapidly inducible upon exposure to IFN-γ, in which STAT-1-responsive GAS motif is essential for maximal IRF-1 expression (Sims *et al.*, 1993). Treatment with LYR-71 attenuated the protein and mRNA levels of IRF-1 as well as inhibiting the promoter activity of the IRF-1 gene in IFN-γ-activated RAW 264.7 cells (Figure 4), indicating that LYR-71 down-regulated IRF-1 induction at the transcription level. The STAT-1-responsive GAS or IRF-1-responsive ISRE motifs have been also identified in the promoter regions of inflammatory genes: iNOS with three GAS sites and two ISRE sites upstream from –722 relative to the transcription start; IP-10 at –224/–212; MIG at –222/–198 and –99/–85 (Lowenstein *et al.*, 1993; Ohmori and Hamilton, 1993; Wong *et al.*, 1994).

As LYR-71 down-regulated IFN-γ-induced expression of inflammatory genes at the transcription level, we tested whether LYR-71 could affect JAK–STAT-1 signalling in macrophages. Supporting the hypothesis, treatment of LYR-71 inhibited IFN-γ-induced transcriptional activity of STAT-1 and also that of IRF-1 in RAW 264.7 cells (Figure 5). In addition, treatment with LYR-71 concentration-dependently reduced STAT-1 binding to the GAS motif in IFN-γ-activated cells (Figure 6A). We next focused on upstream STAT-1 signalling of tyrosine phosphorylation events proximal to the receptor. Treatment with LYR-71 inhibited IFN-γ-induced tyrosine phosphorylation of STAT-1 (Figure 6B) as well as transphosphorylation of JAK-1 and JAK-2 in the cells (Figure 6C). Therefore, LYR-71 could primarily inhibit the tyrosine phosphorylation of JAKs and STAT-1 in response to IFN-γ, sequentially preventing the DNA binding and transcriptional activity of STAT-1 in macrophages.

To elucidate a molecular target involved in the action of LYR-71 on JAK–STAT-1 activation, we first considered catalytic activity of JAK-1 or -2. Treatment with LYR-71 of cell-free enzymes inhibited *in vitro* autophosphorylation of JAK-2 but not that of JAK-1 (Figure 7A). This result would support inhibitory effects of LYR-71 on JAK-2 and also JAK-1 activation via trans-phosphorylation in IFN-γ-stimulated macrophages shown in Figure 6C. Therefore, direct inhibition of JAK-2 activity could provide a molecular basis of the action of LYR-71, uncoupling JAK–STAT-1 activation and sequentially down-regulating the expression of inflammatory genes in IFN-γ-activated macrophages. Similarly, an inhibition of JAK-2 activity by AG-490 prevented IFN-γ-induced NO production in primary macrophages-derived from bone marrows of C3H/HeJ (TLR-4$^{-/-}$) mice (Figure 2B) and also suppressed IFN-γ-induced protein levels of iNOS in RAW 264.7 cells (Figure 3A).

Cellular levels of Tyr^{701}-phosphorylated STAT-1 in response to IFN-γ are also controlled by PTPase-mediated dephosphorylation process, balancing with JAKs-catalysed kinase activity (Wu *et al.*, 2002; Yamada *et al.*, 2003). Treatment with peroxyvanadate, a PTPase inhibitor, significantly potentiated IFN-γ-induced NO production and STAT-1 phosphorylation (Figure 7B,C), indicating the homeostatic balance is indeed existing in RAW 264.7 cells. To determine whether the action of LYR-71 could be dependent on PTPase activity, the cells were treated with LYR-71 in the absence or presence of peroxyvanadate, and then stimulated with IFN-γ. Interestingly, the inhibitory action of LYR-71 on IFN-γ-induced NO production and STAT-1 phosphorylation was consistently diminished in the presence of peroxyvanadate (Figure 7B,C). Further, total cellular PTPase activity was significantly increased upon exposure to LYR-71 alone, and this action was also abolished in the presence of peroxyvanadate (Figure 7D). These results indicate that PTPase-dependent tyrosine dephosphorylation processes can be also included in the action of LYR-71, uncoupling JAK–STAT-1 signalling. Even though PTPases such as SHP-1 and -2 catalyse tyrosine dephosphorylation of STAT-1 and function as negative regulators of IFN-γ-induced NO production (Diaz-Guerra *et al.*, 1999; Wu *et al.*, 2002), we do not know which specific PTPases are involved in the action of LYR-71. This will be the subject of another study in near future.

To date, very few pharmacological agents have been reported to target the activation of PTPases for suppression of IFN-γ-induced JAK–STAT-1 signalling. In particular, curcumin induces the activation of SHP-2 but not that of SHP-1, and also increases SHP-2's association with JAKs in brain microglial cells (Kim *et al.*, 2003). This mechanism confers curcumin's action, down-regulating the expression of iNOS and cyclooxygenase-2 genes in the IFN-γ-activated cells (Kim *et al.*, 2003). Ganglioside increases membrane raft-mediated association of SHP-2 with JAK-2 in brain microglial cells, and also induces the activation of SHP-2, resulting in suppression of JAK–STAT signalling (Kim *et al.*, 2006). *Leishmania* EF-1α is an activator of SHP-1, and attenuates IFN-γ-induced expression of iNOS gene in macrophages (Nandan *et al.*, 2002).

In conclusion, this study clearly demonstrated that LYR-71 could uncouple the tyrosine phosphorylation of JAKs and STAT-1 in response to IFN-γ, sequentially down-regulating the expression of pro-inflammatory genes in macrophages. The molecular basis of LYR-71's action appears to be a direct inhibition of JAK-2 activity, with another action on PTPases sensitive to peroxyvanadate.

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Conflict of interest

The authors state no conflict of interest.

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