ORIGINAL ARTICLE

Studies on novel HIF activators, A-503451s II: biological activities of A-503451A

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In the course of our screening, we discovered a novel compound, A-503451A, as a potent hypoxia-inducible factor (HIF) activator. In human hepatocarcinoma HepG2 cells, A-503451A induced HIF-mediated luciferase reporter gene expression and stabilized HIF-1 α protein. A-503451A increased the mRNA expression levels and the protein secretion of HIF-dependent genes, vascular endothelial growth factor and erythropoietin. Addition of excess ferric chloride to the culture medium suppressed the HIF-induction activity of A-503451A. A-503451A did not have iron-chelating activity *in vitro*, but decreased the intracellular labile iron pool concentration. These data indicate that A-503451A is a unique HIF activator.

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INTRODUCTION

Oxygen is essential for animal life. To tolerate low oxygen conditions (hypoxia), there are several systems that protect cells from such conditions. Hypoxia-inducible factor (HIF) is a central factor in the hypoxic response.^{1–3} HIF is a heterodimeric transcription factor composed of an α -subunit and a β -subunit (HIF-1 β).^{1–2} There are three α -subunits: HIF-1 α , HIF-2 α and HIF-3 α . The functions of HIF-1 α have been studied more extensively than those of HIF-2 α and -3 α . The activity of HIF-1 is mainly controlled by the stabilization of α -subunit. Under normoxic conditions, HIF-1 α is prolyl hydroxylated by three HIF prolyl hydroxylase isoforms (PHD1–3) and then ubiquitinated by the von Hippel–Lindau tumor suppressor gene product (pVHL).^{4–5} Ubiquitinated HIF-1 α is rapidly degraded by the ubiquitin–proteasome pathway. Under hypoxic conditions, the activity of PHD is suppressed, leading to the accumulation of HIF-1 α and subsequent activation of HIF-1 activity. Activated HIF induces multiple hypoxic responses including erythropoiesis, angiogenesis, adaptation to anerobic metabolism and cell survival through the induction of hypoxia-inducible genes (oxygenes).^{6–9} One oxygen is erythropoietin (EPO), which is the main stimulator of erythropoiesis. Recombinant EPO protein is widely used to treat patients suffering from anemia. However, the patients' quality of life remains to be improved because use of recombinant EPO is restricted by its high price and the necessity of i.v. administration. Thus, small MW compounds that can be administered orally and increase EPO would be useful for the treatment of anemia.

From these view points, HIF activators have been intensively screened from a natural and synthetic origin for anti-anemia drug.^{10–11} Most of the HIF activating agents from natural origin are an iron chelator.^{10–16} Iron chelator inhibit PHDs and cause HIF

stabilization and activation.¹⁷ Recently, another type of HIF activators that inhibit PHDs by competing with the natural substrate, 2-oxoglutarate to bind to the active site of PHD have been found through various approaches such as an affinity selection MS technique, structure-based drug design and dynamic-combinatorial MS, and some of them are in clinical development.¹¹ Although understanding of the mechanisms underlying EPO production by HIF have been greatly enhanced, the precise mechanism regulating the HIF activity have not fully uncovered. Therefore, acquiring novel compounds which activate HIF through new mechanisms other than iron chelation and 2-oxoglutarate competition would be useful not only to develop new medication to treat anemia but also to uncover the unknown biological mechanisms in HIF regulation.

We screened microbial metabolites and found a novel compound, A-503451A, as a HIF activator. In a preceding paper, we described the taxonomy of the producing strain, SANK 60101, and the fermentation, isolation, physico-chemical properties and structure elucidation of A-503451A, -B, -C and -D.¹⁸ In this paper, we report the biological activities of A-503451A.

MATERIALS AND METHODS

Materials

Medium, serum and supplements were purchased from Invitrogen (Waltham, MA, USA). Desferrioxamine (DFX) was from Sigma (St Louis, MO, USA). Calcein and its acetoxymethyl ester (calcein-AM) were from Molecular Probes (Waltham, MA, USA).

Cell culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 units ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin and 10% fetal bovine serum. The cells were maintained in a humidified 95% air/5%

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CO₂ incubator at 37 °C. Hypoxic incubations (1% O₂) were performed using a CO₂/multi gas incubator (ACI-165D, ASTEC, Fukuoka, Japan).

Luciferase reporter gene assay

pGVB2-EPO luciferase reporter gene plasmid was constructed by inserting the 126-bp 3' EPO enhancer (corresponds to nucleotides 120 to 245 on the 3' side of the poly (A) addition site) and the 144-bp minimal EPO promoter (corresponds to nucleotides -118 to +26 relative to the transcription initiation site) in the upstream of the firefly luciferase of pGVB2 (Wako, Osaka, Japan). HepG2 cells were seeded at 1.0×10^4 cells per well in 96-well plates. After overnight incubation, HIF-dependent reporter gene plasmid pGVB2-EPO¹⁸ or control reporter gene plasmid pGL3-Control (Promega, Madison, WI, USA) was transfected into HepG2 cells using Tfx-20 reagent (Promega) following the manufacturer's instructions. The following day, the medium was changed to fresh medium containing various concentrations of test samples and maintained under normoxic or hypoxic conditions. After 16-h incubation, the luciferase activity was determined with the use of the Steady-Glo Luciferase Assay System (Promega) following the manufacturer's instructions.

Western blot analysis

HepG2 cells were seeded 1 day before the experiments. The following day, the medium was changed, and if applicable, the cells were preincubated with 100 μ M ferric chloride for 30 min. The cells were stimulated with 100 ng ml⁻¹ (284 nM) A-503451A, 100 μ M DFX or hypoxia for specified times. Then, the cells were lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, protease inhibitor cocktail, pH 7.4), sonicated and centrifuged (15 000 g, 30 min). Proteins were resolved on 4–12% gradient SDS-polyacrylamide gels and blotted onto nitrocellulose membrane. HIF-1 α protein was detected using the anti-HIF-1 α antibody (BD Transduction Laboratory, Haryana, India). Then the membranes were reblotted with anti-HIF-1 β antibody (Cell Signalling Technologies, Danvers, MA, USA) or anti- α -tubulin antibody (Sigma). Images were obtained by Amersham Imager 600 (GE, Tokyo, Japan).

Quantitative RT-PCR

HepG2 cells were seeded at 1.0×10^5 cells per well in six-well plates. After overnight incubation, the medium was changed and the test samples were added. The cells were incubated for 16 h in normoxic or hypoxic conditions and total RNA was isolated using Sepazol (Nacalai Tesque, Kyoto, Japan). The reverse transcription was completed with RT-PCR kit (Takara Bio, Shiga, Japan) using random hexamer primers. Real-time PCR was performed on an ABI Prism 7700 using the TaqMan Universal PCR Master kit (PE Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions.

Expression levels were normalized to 18S ribosomal RNA. Primer pairs were as follows: 5'-CTTGCTGCTCTACCTCCACCAT-3' and 5'-ATGTCCACCAGGGTCTCGATT-3' for vascular endothelial growth factor (VEGF); 5'-GCATGTG GATAAAGCCGTCAGT-3' and 5'-AGAGTTTGC GGAAAGTGCAGC-3' for EPO; 5'-AACGCCAAAGCCACTTCGA-3' and 5'-AGTGCTTCCATCGGAAG GACTAG-3' for HIF-1 α ; and 5'-CATTGGCAGATTGCAGGTAAGT-3' and 5'-ACAGCGGTGATCCACAAAAGT-3' for HIF-1 β . TaqMan probes were as follows: 5'-CACCAAGGCCAGCACATAGGAGAGAGATGA-3' for VEGF; 5'-TCAGCTGCTCCACTCCGAACAATCAC-3' for EPO; 5'-ACCATGCCCC AGATTGAGGATCAGACA-3' for HIF-1 α ; and 5'-CAACCAACAGAGTTCA TCTCCCGACACAAC-3' for HIF-1 β . Pre-developed TaqMan assay reagent Eukaryotic 18S rRNA (Applied Biosystems) was used for 18S rRNA.

ELISA assay

HepG2 cells were seeded at 5.0×10^3 cells per well in 96-well plates. After the overnight incubation, the cells were washed with Dulbecco's modified Eagle's medium two times and test samples were added. The cells were incubated for 16 h in normoxic or hypoxic conditions and the culture supernatant was used for ELISA. ELISA was performed using VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) or EPO ELISA kit (Stemcell Technologies, Vancouver, BC, Canada) following the manufacturer's instructions.

Iron-chelating assay

In vitro iron-chelating activity was measured using the iron-sensing fluorescent probe, calcein. Calcein binds to both iron (II) and iron (III), and this results in quench of its fluorescence.¹⁹ For cell-free assay, one volume of three-fold concentrated test compounds was incubated with the same volume of 3 μ M ferric chloride for 10 min at room temperature, then one volume of 750 nM calcein was added. After 5 min of incubation, the calcein fluorescence signal (excitation = 485 nm; emission = 530 nm) was measured. For cell-based assay, confluent HepG2 cells were treated with A-503451A (284 nM, 1421 nM) or DFX (100 μ M) for 6 h before the addition of 0.4 μ M calcein-AM. After 10 min incubation at room temperature, the cells were washed with PBS two times and the calcein fluorescence signal was measured.

RESULTS

A-503451A induced HIF-dependent reporter gene expression in HepG2 cells

In a preceding paper,¹⁸ we screened microbial metabolites and found a novel compound, A-503451A (Figure 1a), as a HIF activator. First, to monitor the HIF-activation activity of A-503451A, the plasmid pGVB2-EPO (Figure 1b) that drives luciferase reporter gene under the control of the 126-bp 3' EPO enhancer and the 144-bp minimal EPO

Figure 1 Activation of HIF-dependent reporter gene expression by A-503451A in HepG2 cells. (a) Chemical structure of A-503451A. (b) HIF-dependent reporter gene construct (pGVB2-EPO) used for the assay. EPO-E, EPO enhancer; EPO-P, EPO promoter; HIF, hypoxia-inducible factor; HRE, HIF response element. (c) Luciferase activities in HepG2 cells following stimulation with indicated concentrations of A-503451A or 100 μ M DFX (desferrioxamine). Means $\pm$ s.d. of three independent experiments. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

promoter was used. The 126-bp EPO enhancer contains one HIF-binding site. To investigate the effects of A-503451A on HIF-dependent gene expression, pGVB2-EPO was transiently transfected into HepG2 cells and treated with increasing concentrations of A-503451A for 16 h. A-503451A dose-dependently increased the luciferase activity (Figure 1c). The strength of induction was almost equal to that of 100 μ M DFX, the iron chelator used to mimic hypoxia (Figure 1c). The induction of the reporter gene expression did not result from nonspecific transcriptional activation as A-503451A had no effects on the luciferase activity of the control plasmid (pGL3-Control; Figure 1c). Incubation of A-503451A with HepG2 cells for 24 h had a slight (about 10%) decrease of the cell viability (data not shown).

Figure 2 Time-dependent accumulation of HIF-1 α protein in HepG2 cells. HepG2 cells were treated with A-503451A (100 ng ml⁻¹), hypoxia (1% O₂), or DFX (100 μ M) for indicated time period. The HIF-1 α , HIF-1 β and α -tubulin protein was detected by western blotting. Representative images of three independent experiments are shown. DFX, desferrioxamine; HIF, hypoxia-inducible factor. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

A-503451A stabilized HIF-1 α protein

The activity of HIF-1 is mainly controlled by the stabilization of the HIF-1 α subunit under hypoxic conditions. Thus, the effects of A-503451A on HIF-1 α protein levels were investigated. A-503451A at the concentration of 100 ng ml⁻¹ induced time-dependent accumulation of HIF-1 α (Figure 2). HIF-1 α protein accumulation started between 1 and 3 h after A-503451A stimulation and HIF-1 α level remained stabilized up to 24 h. The same accumulation pattern was observed after DFX stimulation, whereas relatively rapid accumulation (within 1 h) was observed after hypoxia stimulation. The protein levels of HIF-1 β subunit were not affected by none of these stimulations (Figure 2).

A-503451A induced HIF-dependent gene expression

The effects of A-503451A on the mRNA expression levels of HIF-target genes were investigated by a quantitative RT-PCR method. Treatment of 500 ng ml⁻¹ A-503451A resulted in the increases of 528 $\pm$ 102% and 285 $\pm$ 45% in the mRNA expression levels of EPO and VEGF, two representative HIF-dependent genes, respectively (Table 1). The level of induction was about half that of hypoxia. A-503451A did not change the mRNA expression levels of HIF-1 α or HIF-1 β , which has been reported the expression levels were not affected by HIF (Table 1). The functionality of A-503451A-induced HIF-dependent gene mRNA expression was further investigated by measuring EPO and VEGF protein in the culture medium of HepG2 cells. Treatment of 500 ng ml⁻¹ A-503451A for 16 h resulted in the increase of 2040 $\pm$ 100% and 246 $\pm$ 3% in the protein secretion levels of EPO and VEGF, respectively (Figures 3a and b).

Table 1 mRNA expression levels of HIF-dependent or -independent genes

	VEGF	EPO	Hif-1 β	HIF-1 α
Normoxia	1.00 $\pm$ 0.47	1.00 $\pm$ 0.30	1.00 $\pm$ 0.35	1.00 $\pm$ 0.24
A-503451A 100 ng ml ⁻¹	4.38 $\pm$ 0.91**	2.74 $\pm$ 1.32	0.77 $\pm$ 0.14	0.78 $\pm$ 0.06
A-503451A 500 ng ml ⁻¹	5.28 $\pm$ 1.02**	2.85 $\pm$ 0.45**	0.77 $\pm$ 0.22	0.70 $\pm$ 0.22
Hypoxia	11.92 $\pm$ 2.27**	6.33 $\pm$ 0.87**	1.04 $\pm$ 0.40	1.74 $\pm$ 0.58
DFX 100 μ M	6.73 $\pm$ 0.54**	2.66 $\pm$ 0.87**	0.53 $\pm$ 0.24	0.76 $\pm$ 0.21 (induction ratio)

Abbreviations: DFX, desferrioxamine; EPO, erythropoietin; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor. Means $\pm$ s.d. of three independent experiments. ** P < 0.01.

Figure 3 Induction of EPO (a) and VEGF (b) protein secretion by A-503451A. HepG2 cells were treated with A-503451A (100 or 500 ng ml⁻¹), hypoxia (1% O₂) or DFX (100 μ M) for 16 h. The EPO or VEGF protein was detected using ELISA. Hyp., hypoxia; Nor., normoxia. Means $\pm$ s.d. of eight or three independent experiments, respectively. ** P < 0.01. DFX, desferrioxamine; EPO, erythropoietin; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

Figure 4 Excess iron ion inhibited the activity of A-503451A. HepG2 cells were pretreated with 200 μ M ferric chloride and then treated with A-503451A (100 ng ml⁻¹ = 284 nM), hypoxia (1% O₂) or DFX (100 μ M). (a) The HIF-1 α , HIF-1 β and α -tubulin protein was detected by western blotting. Representative images of three independent experiments are shown. (b) pGVB2-EPO reporter gene activity. Means $\pm$ s.d. of six independent experiments. DFX, desferrioxamine; DMSO, dimethyl sulfoxide; EPO, erythropoietin; Fe, pretreatment of 200 μ M ferric chloride; HIF, hypoxia-inducible factor. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Figure 5 A-503451A decreased intracellular labile iron pool. (a) *In vitro* iron-chelating activity assay. Means $\pm$ s.d. of four independent experiments. (b) Cell-based calcein-AM test. Means $\pm$ s.d. of 12 independent experiments. ** P < 0.01. DFX, desferrioxamine; DMSO, dimethyl sulfoxide.

Excess iron ion prevented the HIF activation by A-503451A

To examine the mechanism of HIF activation by A-503451A, the effects of iron ion, a known HIF inhibitor, on the activity of A-503451A were investigated. PHD requires both iron ion and oxygen for its activity that is prolyl hydroxylation of HIF-1 α and resulting degradation of HIF-1 α and inactivation of HIF.¹⁷ Iron chelators are known to activate HIF through the inhibition of PHDs. The addition of excess ferric chloride (200 μ M) to the culture medium suppressed the HIF-1 α accumulation induced by A-503451A and DFX (Figure 4a). Ferric chloride did not suppress the HIF-1 α accumulation induced by hypoxia (Figure 4a). In accordance with these results, the activity of A-503451A and DFX on a HIF-dependent reporter was also suppressed by ferric chloride, though that of hypoxia was not affected (Figure 4b). To examine the possibility that A-503451A activates HIF by chelating iron ion like DFX, the iron-chelating activity of A-503451A was measured using the iron-sensing fluorescent probe, calcein. Calcein binds to both iron (II) and iron (III), and this results in quench of its fluorescence.¹⁹ Incubation of calcein with 1 μ M ferric chloride resulted in the quench of calcein signal (Figure 5a). Reversal of the quenched calcein signal by the addition of a test compound gives an indication that the compound has an iron-chelating activity. A-503451A had no effect on the calcein signal while DFX dose-dependently restored the quenched calcein signal (Figure 5a). Next, the effects of A-503451A on the intracellular labile iron pool concentration were examined using the calcein-AM test.²⁰ A-503451A restored the quenched calcein signal as DFX (Figure 5b). These

findings demonstrated that A-503451A, though it did not function as an iron chelator, affected the intracellular labile iron pool concentration.

DISCUSSION

In the course of screening studies for HIF activators, we discovered a novel compound, A-503451A, as a potent HIF activator. Exposure to A-503451A resulted in the accumulation of HIF-1 α protein and upregulation of oxygenes such as VEGF and EPO in HepG2 cells even under normoxic conditions.

The induction of HIF-dependent genes by A-503451A was about 50% of that of hypoxia (Figure 3, Table 1). Even treatment of relatively high concentration (over 1 μ g ml⁻¹) of A-503451A did not reach the same induction level as that of hypoxia, indicating that A-503451A did not induce the whole set of HIF activation processes caused by hypoxia. It has been reported that hydroxylation, phosphorylation and acetylation of HIF-1 α regulate the stability and activity of HIF-1 α .^{4,21–23} The accumulation level of HIF-1 α protein by A-503451A was equal to that by hypoxia (Figure 2), indicating the modifications affecting HIF activity was not fully occurred by A-503451A. Nevertheless, A-503451A is capable of inducing EPO and VEGF protein to a level comparable to that by hypoxia in HepG2 cells, suggesting A-503451A targets a critical step in HIF activation.

The von Hippel–Lindau tumor suppressor gene product (pVHL), a part of the E3 ubiquitin protein complex, binds to HIF-1 α , resulting in the ubiquitination and degradation of HIF-1 α through the

ubiquitin–proteasome pathway.⁵ Hydroxylation of Pro-402/564 in the region of HIF-1 α known as the oxygen-dependent degradation domain is required for the binding of HIF-1 α and pVHL and PHD hydroxylates the proline residues in oxygen-dependent degradation domain.⁴ The enzymatic activity of PHD depends on iron and oxygen. DFX activates HIF-1 through chelating iron and inhibiting hydroxylation of both the proline and asparagine residues of HIF-1 α . Suppression of the activity of A-503451A by excess ferric chloride suggested A-503451A activated HIF by limiting iron utilization. Its chemical property¹⁸ and the results shown in Figure 5 indicate that A-503451A does not function as iron chelator, but acts on an unidentified target that can be overridden by excess iron ion. Decrease of intracellular labile iron pool concentration by A-503451A indicates that one possible target is intracellular iron transport system. Most of HIF activators discovered from natural origins activate HIF through iron-chelating activity.^{10–16} Therefore, unique HIF activation mechanism by A-503451A would provide valuable clues to understand biological mechanisms regulating HIF activity.

It has also been reported that HIF can be accumulated and activated during normoxia by growth factor or cytokines.^{24–26} The involvement of the PI-3 kinase pathway, p42/44 MAP kinase pathway and nuclear factor-kappa B pathway has been reported. PI-3 kinase inhibitor, LY294002, p42/44 MAP kinase inhibitor, PD98059 or nuclear factor-kappa B signal inhibitor, SN50, did not suppress the activity of A-503451A (data not shown). Therefore, neither the PI-3 kinase, p42/44 MAP kinase nor nuclear factor-kappa B pathway was involved in the HIF activation by A-503451A. Further studies are necessary to identify the mechanism of the HIF activation by A-503451A.

In conclusion, we have isolated novel compound A-503451A that activates HIF under normoxic conditions. A-503451A increased the mRNA and protein expression of oxygenes, and induced HIF-1 α protein accumulation. Better understanding of the induction mechanism may help in the elucidation of hypoxia-regulated gene expression and provide a novel molecular target for development of pharmaceutical agents.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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