Vesnarinone Suppresses TNFα mRNA Expression by Inhibiting Valosin-Containing Protein

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ABSTRACT

Vesnarinone is a synthetic quinolinone derivative used in the treatment of cardiac failure and cancer. It is also known to cause agranulocytosis as a side effect, which restricts its use, although the mechanism underlying agranulocytosis is not well understood. Here, we show that vesnarinone binds to valosin-containing protein (VCP), which interacts with polyubiquitinated proteins and is essential for the degradation of IκBα to activate nuclear factor (NF)κB. We show that vesnarinone impairs the degradation of IκBα, and that the impairment of the degradation of IκBα is the result of the inhibition of the interaction between VCP and the 26S proteasome by vesnarinone. These results suggest that vesnarinone suppresses NFκB activation by inhibiting the VCP-dependent degradation of polyubiquitinated IκBα, resulting in the suppression of tumor necrosis factor-α mRNA expression.

Introduction

Vesnarinone (3,4-dihydro-6-[4-(3,4-dimethoxy-benzoyl)-1-piperazinyl]-2(1H)-quinolinone) is a quinolinone derivative developed as an inotropic agent for the treatment of congestive heart failure (CHF) (Cavusoglu et al., 1995) to modulate Ca2+ channels (Yatani et al., 1989). Vesnarinone is now known to have other activities, such as immunosuppressive activity (Matsui et al., 1994; Sato et al., 1995), the inhibition of human immunodeficiency virus production, the reduction of endotoxemic lethality, and the suppression of the growth of various tumor cell lines, including gastric cancer, lung cancer, adenoid squamous carcinoma, and myeloid leukemia (Fujiwara et al., 1997; Nio et al., 1997; Honma et al., 1999; Kubo et al., 1999; Yokozaki et al., 1999). However, the induction of agranulocytosis has been reported as a side effect of vesnarinone, thereby representing a limitation on its use (Cohn et al., 1998).

At the molecular level, vesnarinone appears to enhance myocardial contractility by augmenting sodium-calcium exchange (Yatani et al., 1989), which may be responsible for the treatment effects in CHF. Vesnarinone is also known to inhibit phosphodiesterase III (PDE3), resulting in an increase in the cyclic AMP concentration in cells, leading to vasodilatation (Itoh et al., 1993). Although these pharmacologic effects may be related to the treatment of CHF, the molecular basis of the side effect is not well understood.

Previously, we showed that vesnarinone impairs the production of tumor necrosis factor alpha (TNFα) in bone marrow stromal cells, an event that is essential for the differentiation of the cells into mature granulocytes (Nabeshima et al., 1997; Hiramoto et al., 2004). These findings are also supported by another study that showed that vesnarinone suppressed both the activation of the transcription factor nuclear factor kappa B (NFκB) and the expression of the TNFα gene, a target of NFκB (Manna and Aggarwal 2000).

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ABBREVIATIONS: CHF, congestive heart failure; FG-EDGE, ferrite glycidyl methacrylate-ethylene glycol diglycidyl ether; NFκB, nuclear factor kappa B; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; siRNA, small interfering RNA; TNFα, tumor necrosis factor alpha; VCP, valosin-containing protein.
These findings raised the possibility that the inhibition of NFκB signaling by vesnarinone may cause the observed agranulocytosis.

NFκB is a key transcription factor that regulates many processes, including the immune response, inflammation, and stress responses. When a cell is not stimulated, NFκB is sequestered in the cytosol through the formation of a complex with a member of the IκB family. However, once the cell is stimulated by factors such as TNFα and interleukin (IL)-1β, the IκB kinase phosphorylates IκB, and phosphorylated IκB is then ubiquitinated and degraded by the proteasome. The released NFκB enters the nucleus and functions as a homo- or heterodimer transcription factor with a member of the NFκB family. Genes related to the immune response, inflammation, and other processes are known to be targets of the NFκB transcription factors.

To investigate the mechanism by which vesnarinone inhibits the activation of NFκB, we attempted to purify a vesnarinone-binding protein with high-performance affinity magnetic beads (Shimizu et al., 2000; Nishio et al., 2008), which are powerful tools for the identification of the molecular targets of many drugs, including thalidomide (Ito et al., 2010). Indeed, a valosin-containing protein (VCP) was identified as a result of our application of this method. It is thought that VCP plays important roles in ubiquitin-dependent protein quality control and intracellular signaling pathways [reviewed in Meyer et al. (2012)].

We further showed that VCP is essential for the ubiquitin-dependent proteasome-mediated degradation of IκBα and that vesnarinone induces the accumulation of ubiquitinated IκBα, resulting in the inhibition of NFκB activation by preventing the interaction between VCP and the 26S proteasome.

Materials and Methods

Plasmid Construction, Antibodies, and Materials. Human VCP and IκBα cDNAs were subcloned from a LP101 cell cDNA library into the mammalian expression vector pHyg-EF-2 (Nishizawa et al., 2003). Vectors encoding the VCP deletion mutants were created by polymerase chain reaction (PCR) using mutagenic primers. Antibodies specific for FLAG (M2, mouse monoclonal; Sigma-Aldrich, St. Louis, MO), VCP (mouse monoclonal; Progen, Heidelberg, Germany), IκBα (sc-371, rabbit polyclonal; Santa Cruz Biotechnology, Dallas, TX), phosphorylated IκBα (Cell Signaling Technology, Danvers, MA), actin (EDM Millipore, Billerica, MA), and ubiquitin (FK2, mouse monoclonal; Biomol (Enzo Life Sciences), Farmingdale, NY) were purchased from the indicated suppliers.

Affinity Purification of Vesnarinone-Binding Proteins Using FG-Beads. FG-EGDE (ferrite glycidyl methacrylate-ethylene glycol diglycidyl ether) beads were prepared as described previously (Nishio et al., 2008). The vesnarinone amino acid derivative was incubated with the FG-EGDE beads in distilled water for 24 hours at 37°C. The vesnarinone amino acid derivative-fixed FG-EGDE beads were washed three times with distilled water and stored at 4°C until use. The LP101 cell membrane extracts (Dignam et al., 1983) were incubated with the vesnarinone amino acid derivative-fixed beads for 4 hours at 4°C, and the beads were then washed three times with binding buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 0.2 mM EDTA, 10% glycerol, 0.1% NP-40, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin A, and 1 μM leupeptin]. The bound proteins were eluted with Laemmli dye or binding buffer containing the vesnarinone amino acid derivative. The eluted proteins were subjected to SDS-PAGE, silver stained, and then subjected to in-gel digestion with trypsin. The peptide fragments were analyzed by quadrupole time-of-flight mass spectrometry, as described previously (Shimizu et al., 2000).

In Vitro Binding Assays. Lysates of 293T cells expressing the VCP mutants were incubated with the vesnarinone amino acid derivative-fixed FG-EGDE for 4 hours at 4°C and washed three times with binding buffer. The bound proteins were eluted with Laemmli dye and subjected to SDS-PAGE, followed by immunoblotting with an anti-FLAG antibody.

Cell Culture, Transfection, and VCP Knockdown. The 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in 5% CO2, and the LP101 cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% fetal calf serum in 5% CO2. The knockdown of VCP in the 293T cells was performed using lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA).

Real-Time Reverse Transcription-Polymerase Chain Reaction. The LP101 cells were treated with 5 ng/ml TNFα for 60 minutes, and the 293T cells were treated with 10 ng/ml TNFα for 60 minutes. The total RNAs were then prepared using Sepasol RNA I Super (Nakalai Tesque, http://www.nacalai.co.jp). The quantification of the TNFα or glyceraldehyde-3-phosphate dehydrogenase mRNA levels was performed using the QuantiTect SYBR Green reverse transcription PCR master mix (Qiagen, http://www.qiagen.com).

Coimmunoprecipitation Assay and Immunoblotting. Lipofectamine 2000 was used to transfect the 293T cells with pHyg-IκBα-His-FLAG or pcDNA-VCP-His-FLAG. At 2 days post-transfection, the cells were treated with 5 μM MG132 for 60 minutes, followed by stimulation with 10 ng/ml TNFα for 20 minutes. The cells were then harvested, washed twice with phosphate-buffered saline (PBS), and lysed with radio-immunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 10 mM N-ethylmaleimide, 20 mM NaF, 25 μM MG132, 1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin] or NP-40 lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% NP-40, 10 mM N-ethylmaleimide, 20 mM NaF, 25 μM MG132, 1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin] at 4°C for 30 minutes. The samples were centrifuged at 20,000g at 4°C for 15 minutes, and the supernatants were incubated with anti-FLAG M2 affinity gel (Sigma-Aldrich) for 2 hours at 4°C. The beads were then washed three times with the same buffer used as the binding buffer, and the bound proteins were eluted with a buffer containing the FLAG peptide (Sigma-Aldrich), subjected to SDS-PAGE, and analyzed by immunoblotting.

Results

Vesnarinone Suppresses the TNFα-Induced Activation of NFκB. Previously, we reported that vesnarinone inhibits the production and the secretion of TNFα from human bone marrow stromal LP101 cells (Nabeshima et al., 1997; Hiramoto et al., 2004), but its molecular mechanisms remained unclear. Based on our previous observations, we first examined whether the inhibition of TNFα secretion in LP101 cells is the result of the reduction of TNFα mRNA expression. After the treatment of LP101 cells with different concentrations of vesnarinone, the cells were induced with TNFα and the quantity of newly transcribed TNFα mRNA was determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). As shown in Fig. 1A, the expression of TNFα mRNA in the LP101 cells was induced by TNFα and reduced by the addition of vesnarinone in a dose-dependent manner. These results suggested that the observed inhibition of TNFα secretion is the result of decreased TNFα mRNA expression caused by the treatment with vesnarinone.

Next, we used human embryonic kidney 293T cells to examine whether the reduction of TNFα mRNA expression by vesnarinone is cell-type specific. A reduction of the induced
TNFα mRNA was also observed in this cell line (Fig. 1B), suggesting that the vesnarinone-induced reduction of TNFα expression is not restricted to bone marrow stromal cells.

As NFκB is known to be the major transcription factor regulating TNFα expression, we next investigated whether vesnarinone inhibits NFκB-dependent transcription. A luciferase gene under the control of four tandem-repeated NFκB binding sites was transfected into the 293T cells, and the effects of vesnarinone on the NFκB-driven reporter gene expression after TNFα treatment was examined. The TNFα-induced expression of the reporter gene was inhibited by the treatment with vesnarinone in a dose-dependent manner (Fig. 1C). These results suggested that vesnarinone inhibited NFκB-dependent transcriptional activation.

It is known that NFκB activity is regulated by specific inhibitory subunits, the IκB proteins, which are degraded by the ubiquitin-proteasome system during NFκB activation. If IκB is not properly degraded, NFκB cannot enter the nucleus and fail to activate NFκB-dependent transcription. To determine whether the inhibitory effect of vesnarinone is due to an effect on IκB degradation, the cytoplasmic level of IκBα was measured by immunoblotting (Fig. 1D). In the absence of vesnarinone, a reduction of IκBα protein was observed after 15 minutes of TNFα treatment as expected and the IκBα signal was recovered by 60 minutes. However, in the presence of vesnarinone, the inhibition of IκBα degradation was observed even after TNFα treatment (Fig. 1D). This result suggested that vesnarinone inhibits the IκBα degradation process.

As it is known that the degradation of IκB is triggered by IκB kinase-mediated phosphorylation, we next examined whether IκB is phosphorylated after TNFα treatment. Interestingly,
although the phosphorylation of IκBα was detected either in the presence or absence of vesnarinone at 15 minutes after the TNFα stimulation, the amount of phosphorylated IκBα rapidly decreased in the absence of vesnarinone, which correlated well with the degradation of IκB (Fig. 1D). Conversely, a substantial amount of phosphorylated IκBα was detected even after TNFα stimulation in the presence of vesnarinone. This result correlated well with the remaining amount of IκBα (Fig. 1D). These results suggested that vesnarinone does not inhibit the phosphorylation of IκBα but does inhibit the degradation of IκB at a specific point between IκB phosphorylation and degradation.

Identification of Vesnarinone-Binding Proteins. To clarify the molecular mechanisms of the vesnarinone-induced inhibition of NFκB activation, we attempted to purify vesnarinone-binding proteins directly using high-performance affinity purification (Shimizu et al., 2000). The amino acid derivative of vesnarinone was immobilized on FG beads (Nishio et al., 2008) via the epoxy group (Fig. 2A) and then used for the purification of vesnarinone-binding proteins. The vesnarinone-fixed beads were incubated with extracts of human bone marrow stromal LP101 cells, and the binding proteins were directly purified. A 97-kDa protein was found to bind specifically to the vesnarinone-fixed beads, and the subsequent quadrupole time-of-flight mass spectrometry analysis identified the 97-kDa protein as a valosin-containing protein (VCP) (Fig. 2B). VCP is a member of the ATPases associated with diverse cellular activities (AAA) and possesses two ATPase domains, and it is known to play a critical role in many cellular activities such as the ubiquitin-proteasome system, endoplasmic reticulum-associated degradation of proteins, cell cycle, and DNA repair (reviewed in Meyer et al., 2012). The binding specificity of VCP to vesnarinone was examined by adding free vesnarinone to the elution buffer (Fig. 2B). The addition of free vesnarinone led to the release of VCP from the beads in a concentration-dependent manner, suggesting that VCP is a vesnarinone-binding protein (Fig. 2B). The identification of VCP as a vesnarinone-binding protein was further confirmed by immunoblotting using an anti-VCP antibody (Fig. 2B), which specifically reacted with the purified 97-kDa protein. In addition, the recombinant VCP (rVCP) protein overexpressed in Escherichia coli also has the ability to bind to the vesnarinone-fixed beads, as shown in Fig. 2C, indicating that vesnarinone binds to directly VCP.

Determination of the Vesnarinone-Binding Region of VCP. We then determined the vesnarinone-binding region of VCP. VCP is known to have two ATPase domains, the D1 and D2 domains, which are followed by the N-terminal region of the polyubiquitin-recognition domain (Dai et al., 1998). Thus, we generated a FLAG-His-tagged full-length version and a series of deletion mutants of VCP that have either one or two ATPase domains and assessed the ability of the full-length and mutant recombinant VCP derivatives to bind to the beads. The full-length and deletion mutants consisting of amino acids 186–806, 1–481 and 149–494, which contain the D1 ATPase domain, bound to the vesnarinone-fixed beads. In contrast, two mutants consisting of residues 454–806 and 1–192, lacking the D1 domain, did not bind to the beads (Fig. 3). These results indicated that vesnarinone binds to the central region of VCP, which corresponds to the D1 ATPase domain. This result raised the possibility that VCP is required for NFκB activation and that the binding of vesnarinone to VCP causes a functional alteration in VCP, resulting in the impairment of NFκB activation.

VCP Is Required for NFκB-Dependent Gene Activation. If vesnarinone inhibits VCP function, then the effect of vesnarinone should be similar to that of VCP malfunction. Thus, we knocked down VCP expression using a VCP-specific small interfering RNA (siRNA) and compared the effect of vesnarinone on the transcriptional activation of NFκB target
A Western blot analysis showed that the specific siRNA reduced the level of VCP protein by >90% at 2–4 days after the siRNA transfection, whereas it had little effect on the protein level of actin (Fig. 4).

After the induction of the cells with TNFα, the expression of the NFκB target genes TNFα, IκBα, and A20 was examined by quantitative PCR. When the VCP expression was knocked down by the VCP-specific siRNA, the induction of TNFα gene expression was significantly attenuated (Fig. 4B), comparable to the result of the vesnarinone treatment (Fig. 1). In addition to the attenuation of TNFα, the induced expression of the other NFκB target genes IκBα and A20 mRNA was also attenuated (Fig. 4, C and D). These results suggest that both vesnarinone treatment and VCP knockdown attenuate the expression of NFκB target genes in TNFα-stimulated cells by affecting NFκB activity.

This result, combined with the finding that phosphorylated IκBα is not degraded but rather accumulated in the presence of vesnarinone or VCP knockdown. Thus, we examined the effect of vesnarinone on the accumulation of ubiquitinated IκBα by performing a transient transfection of a FLAG-IκB expression vector in 293T cells. The cells were incubated with various concentrations of vesnarinone, followed by FLAG-immunoprecipitation in a highly stringent buffer to purify and concentrate the ubiquitinated IκBα, which was detected by immunoblotting using antiubiquitin or anti-IκBα antibodies.

Predictably, the high molecular weight ubiquitinated protein detected using the antiubiquitin antibodies and the high molecular weight IκBα detected using the anti-IκBα antibodies were both enhanced in a dose-dependent manner by the treatment with vesnarinone (Fig. 5A, lanes 3–6). In addition, these signals produced similar pattern to the MG132 treatment (Fig. 5A, lane 7). Thus, we concluded that ubiquitinated IκBα accumulated after the vesnarinone treatment.

As the knockdown of VCP showed similar effects to vesnarinone, we examined whether the knockdown of VCP can also cause the accumulation of polyubiquitinated IκBα. As determined by Western blotting, the knockdown of VCP induced the accumulation of ubiquitinated IκBα (Fig. 5B). These results suggest that VCP is significant for the degradation of IκBα and the activation of NFκB.

Vesnarinone Prevents the Interaction between VCP and the 26S Proteasome. Because VCP is known to bind to ubiquitinated proteins such as IκBα, cyclin E, and hypoxia-inducible factor (HIF)1α (Dai et al., 1998; Yen et al., 2000; Dai and Li, 2001; Asai et al., 2002; Alexandru et al., 2008; Cayli et al., 2009; ) and contributes to their degradation, we examined whether vesnarinone inhibits the interaction of
VCP with ubiquitinated IκBα or the 26S proteasome. An expression vector encoding VCP-His-FLAG was transfected into 293T cells, and a coimmunoprecipitation assay was performed using an anti-FLAG antibody in the presence of MG132. As shown in Fig. 6A, in the presence of MG132, 20S C2, which is a component of the 26S proteasome, was coimmunoprecipitated with VCP-His-FLAG (Fig. 6A, lane 4), whereas 20S C2 was not precipitated without MG132 (Fig. 6A, lane 3). Interestingly, the quantity of the coprecipitated 20S C2 was reduced by increasing the amount of vesnarinone. These results suggested that vesnarinone inhibits the interaction between FLAG-tagged VCP and proteasomal 20S C2 (Fig. 6A, lanes 5–7).

When FLAG-IκBα-expressing cells were used for the coimmunoprecipitation assay, both VCP and 20S C2 were coprecipitated. In the presence of vesnarinone, the quantity of 20S C2 was reduced in a dose-dependent manner, whereas the quantity of VCP was unchanged. As the total amount of ubiquitinated IκB in the assay was almost the same, it can be concluded that vesnarinone inhibited the interaction between VCP and the 26S proteasome but not the interaction between VCP and ubiquitinated IκB.

Although we had tried to analyze the interaction between VCP and endogenous 20S C2 by coimmunoprecipitation using anti-20S C2 antibody, we failed to detect VCP or IκB with 20S C2, probably because endogenous proteasomal proteins expression was so abundant, and content of the VCP-interacted 20S C2 was quite limited in mammalian cells.

While coimmunoprecipitation assay is limited to show the interaction between VCP and ubiquitinated IκB, these results suggested that ubiquitinated IκB could interact with 20S C2 by mediating with VCP and that vesnarinone blocks proteasomal degradation of the ubiquitinated IκB by inhibiting the interaction between VCP and 20S C2.

Discussion

In this study, we showed that one of the molecular targets of vesnarinone is VCP (valosin-containing protein, also known as p97 and cdc48 in yeast), a member of the AAA ATPase family (Dai and Li, 2001). VCP is known to bind ubiquitinated proteins, such as IκBα, cyclin E, and hypoxia-inducible factor (HIF)1α and to contribute to the ubiquitin-dependent proteasome-mediated degradation of proteins (Dai et al.,...
1998; Yen et al., 2000; Dai and Li, 2001; Asai et al., 2002; Alexandru et al., 2008; Cayli et al., 2009). Vesnarinone induced the accumulation of ubiquitinated I\(_{\kappa}B_a\) by inhibiting the interaction between VCP and the 26S proteasome, which was essential for the degradation of I\(_{\kappa}B_a\) and the activation of NF\(_{\kappa}B\), implying that vesnarinone inhibited the function of VCP.

It has been reported that ubiquitinated I\(_{\kappa}B_a\) remained bound to the p65-containing complexes in cells treated with a proteasome inhibitor, which also supports our results and others (Didonato et al., 1996; Roff et al., 1996). These results suggested that vesnarinone is an NF\(_{\kappa}B\) pathway inhibitor and that vesnarinone suppresses the activation of NF\(_{\kappa}B\) by inhibiting the interaction of VCP with the 26S proteasome. As NF\(_{\kappa}B\) is an essential transcription factor for TNF\(_{\alpha}\) activation, it is reasonable that the failure of NF\(_{\kappa}B\) activation by vesnarinone directly affects TNF\(_{\alpha}\) activation. This new finding can explain our previous data and other results (Manna and Aggarwal 2000) that showed vesnarinone inhibits the TNF\(_{\alpha}\) activation in HL60 and other cell lines. Although it was shown that vesnarinone inhibited the TNF\(_{\alpha}\) expression by inhibiting NF\(_{\kappa}B\) activation in a previous study (Manna and Aggarwal 2000), we could explain this inhibition based on molecular mechanisms, showing that vesnarinone inhibits the I\(_{\kappa}B\) degradation mediated by VCP, which is a novel molecular target of vesnarinone. One of the important differences between the present and previous study is the I\(_{\kappa}B\) phosphorylation status following vesnarinone treatment. While the previous study showed inhibition of I\(_{\kappa}B\) phosphorylation by vesnarinone, we could observe I\(_{\kappa}B\) phosphorylation even after vesnarinone treatment. The difference may result from the difference in the cell lines, but our finding revealed another mechanism of NF\(_{\kappa}B\) regulation by vesnarinone.

Vesnarinone is an inotropic agent for the treatment of congestive heart failure with several known modes of action. For example, vesnarinone is known to augment sodium-calcium exchange (Yatani et al., 1989), resulting in enhanced myocardial contractility; to inhibit phosphodiesterase III (PDE3), resulting in an increase of the cyclic AMP concentration; to increase intracellular calcium ions; to alter sodium and potassium channels; and to activate the phosphorylation of cell adhesion-related molecules. Our results presented here add a new role for vesnarinone to the above list of effects.

Our study also provides new insight into the regulation of NF\(_{\kappa}B\) activity. As NF\(_{\kappa}B\) is an important transcription factor in immune responses, inflammation, cell proliferation, and other important biologic processes, it has been considered an important target for drug development. Many small molecules have been reported to be inhibitors of NF\(_{\kappa}B\) through their effects on the NF\(_{\kappa}B\) activation process.

One group of small molecule inhibitors targets the activity of NF\(_{\kappa}B\). For example, gallic acid is reported to interfere with the binding activity of p65 (Choi et al., 2009). Proteasome inhibitors, such as PS-341 and PS-519, are known to inhibit
protease activity directly (Sunwoo et al., 2001). In addition to the direct inhibition of the proteasome, the protein degradation pathway prior to proteasome entry is also a target of many compounds. For example, benzoquinones and herbimycin are known inhibitors of IkB kinase activity (Ogino et al., 2004) and sesquiterpene lactones are also hypothesized to interfere with the IkB kinase. Because vesnarinone is supposed to inhibit the interaction between VCP and a proteasome component, it can be placed in a new category, and at present no chemicals are known to have the same activity. Thus, our study raises the possibility of regulating the NFkB activity by a new target molecule and VCP can be considered a novel target of anti-inflammatory and immune drugs.

Recently, multiple functions of VCP have been identified (Meyer et al., 2012), including autophagy (Ju et al., 2009), endolysosomal sorting and regulation of proteins (Ritz et al., 2011), mitochondrial membrane protein turnover (Braun et al., 2006), and genome stability (Meerang et al., 2011). Although we only suggested the inhibition of the interaction between VCP and the 26S proteasome by vesnarinone in terms of NFkB inactivation, vesnarinone might affect many other biologic processes within other cellular contexts. Indeed, the examination of the function of vesnarinone via VCP within the context of different cells is an intriguing avenue of study. For example, as a previous study suggests that VCP is required for the degradation of cyclin E, a cell cycle regulator (Dai and Li, 2001), vesnarinone may influence the proliferation of cancer cells by regulating the degradation of cyclin E. In fact, vesnarinone inhibits the growth of several cancer cell lines (Honma et al., 1999; Yokozaki et al., 1999), raising the possibility that vesnarinone might have antitumor activity by affecting the function of VCP and the degradation of cyclin E. As VCP is known to have multiple functions, the refined regulation of VCP may be useful in the development of drugs that will be used for aspects other than NFkB-related processes.

In this study, we used a high-performance affinity chromatography protocol developed in our laboratory (Shimizu et al., 2000) and identified VCP as a vesnarinone-binding protein. We have previously shown that several drug targets, including thalidomide, can be identified efficiently (Ito et al., 2010) by using this technique. The identification of VCP as a molecular target of vesnarinone might have been difficult without this technique.

**Authorship Contributions**

**Participated in research design:** Kabe, Aizawa, Imai, Yamaguchi, Handa.

**Conducted experiments:** Hotta, Nashimoto, Yasumura, Suzuki, Azuma, Iizumi, Shima, Nabeshima, Hiramoto, Okada, Sakata-Sogawa, Tokunaga, Ando, Sakamoto.

**Wrote or contributed to the writing of the manuscript:** Hotta, Ito, Watanabe, Handa.

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