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Discovery of a Small Molecule PDI Inhibitor That Inhibits Reduction of HIV-1 Envelope Glycoprotein gp120

Maola M. G. Khan,[†,‡] Siro Simizu,[†,§] Ngit Shin Lai,[†,¶] Makoto Kawatani,[†] Takeshi Shimizu,[†] and Hiroyuki Osada[†,‡,*]

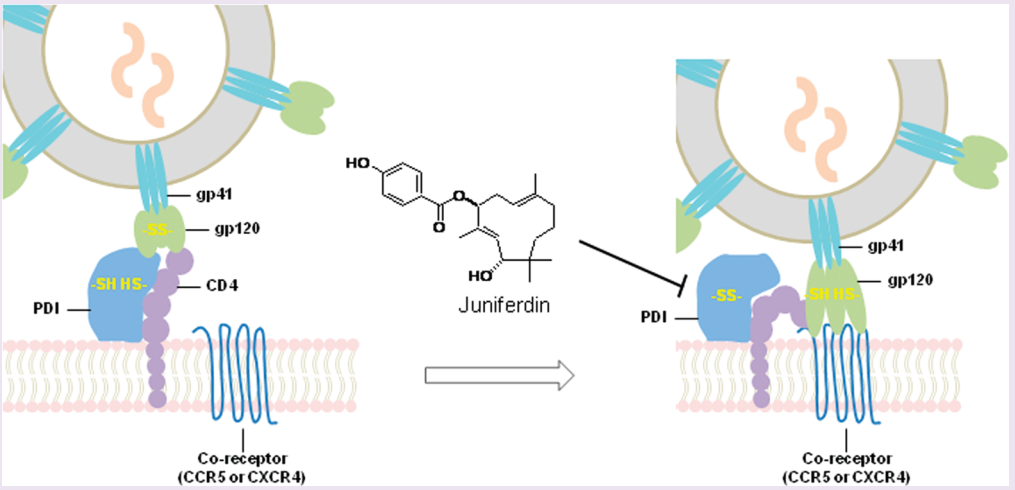
[†]Chemical Library Validation Team, Chemical Biology Core Facility, Chemical Biology Department, RIKEN Advanced Science Institute, Saitama, Japan

[‡]Graduate School of Science and Engineering, Saitama University, Saitama, Japan

[§]Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan

Supporting Information

ABSTRACT: Protein disulfide isomerase (PDI) is a promiscuous protein with multifunctional properties. PDI mediates proper protein folding by oxidation or isomerization and disrupts disulfide bonds by reduction. The entry of HIV-1 into cells is facilitated by the PDI-catalyzed reductive cleavage of disulfide bonds in gp120. PDI is regarded as a potential drug target because of its reduction activity. We screened a chemical library of natural products for PDI-specific inhibitors in a high-throughput fashion and identified the natural compound juniferdin as the most potent inhibitor of PDI. Derivatives of juniferdin were synthesized, with compound **13** showing inhibitory activities comparable to those of juniferdin but reduced cytotoxicity. Both juniferdin and compound **13** inhibited PDI reductase activity in a dose-dependent manner, with IC_{50} values of 156 and 167 nM, respectively. Our results also indicated that juniferdin and compound **13** exert their inhibitory activities specifically on PDI but do not significantly inhibit homologues of this protein family. Moreover, we found that both compounds can inhibit PDI-mediated reduction of HIV-1 envelope glycoprotein gp120.



Protein disulfide isomerase (PDI) is a 57-kDa oxidoreductase of the thioredoxin superfamily that is expressed mainly in the endoplasmic reticulum (ER) of eukaryotic cells. In the ER, PDI acts predominantly as an oxidase to form disulfide bonds in nascent secretory proteins. Furthermore, it catalyzes the rearrangement of incorrect disulfide bonds through isomerase activity, thus mediating proper protein folding.[1,2] PDI is also expressed on the cell surfaces of lymphocytes, hepatocytes, and platelets[3,4] and, together with additional ER secretory proteins, can be secreted to the cell surface through a regulatory pathway. In endosomes and on the cell surface, PDI acts as a reductase to cleave disulfide bonds in proteins.[5−7] Surface-associated PDI is involved in a wide range of functions, including the reduction of disulfide bonds in diphtheria toxin, facilitating the entry of the toxin into cells, the reduction of disulfide bonds in the ectodomain of human thyrotropin receptor, the maintenance of plasma membrane reductive status, and nitric oxide signaling in megakaryocytes and platelets.[8]

The functions of PDI are regulated by the redox state of its active site *CXXC* motif, Cys-Gly-His-Cys in PDI. In this motif, the two amino acids lying between the cysteine residues are important in maintaining its redox potential. All PDI-catalyzed reactions occur through thiol-disulfide exchanges between PDI and neighboring proteins or peptides. During oxidation, PDI with an oxidized *CXXC* motif reacts with the substrate dithiols of proteins or peptides to form disulfide bonds. During reduction, however, PDI with a cysteinyl dithiol *CXXC* motif reduces the

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disulfide bonds of substrate proteins or peptides, resulting in an active-site disulfide in PDI.[9]

The reductase activity of surface-associated PDI is of immense importance because of its involvement in HIV-1 fusogenic events.[10−14] PDI clusters in the vicinity of the HIV-1 primary receptor CD4, which has separate binding sites for PDI and the HIV-1 envelope glycoprotein gp120.[15] The gp120 of HIV-1 binds to CD4, followed by the PDI-catalyzed reduction of at least two of the nine disulfide bonds of gp120. Reduction of the structure-stabilizing disulfide bonds of gp120 leads to major conformational changes, enhancing the ability of gp120 to interact with its co-receptors CCR5 and CXCR4.[10,16] Subsequently, gp41, a viral glycoprotein that is noncovalently bound to gp120, undergoes rearrangement into its fusogenic intermediates and HIV-1 enters into the host cell.[13,16] We have sought to therapeutically target the PDI-mediated rearrangement of gp120 using small molecule inhibitors of PDI.

Due to the ubiquitous role of PDI, it is difficult to target a specific function of the enzyme. Potential therapeutic agents must be unable to permeate cell membranes to specifically inhibit the reductase activity of PDI on the cell surface. If the inhibitor is membrane-permeable, it will inhibit the oxidase function of PDI, which is important for the proper protein folding. Several agents, such as bacitracin, DTNB, pCMBS, PAO, aPAO, GSAO, diethylstilbesterol, and estrone, have been described as PDI inhibitors, although none has been used therapeutically.[10,15,17] Bacitracin was one of the first agents used as a PDI inhibitor, but its clinical use is limited by nephrotoxicity,[18] low membrane permeability,[19] and PDI-independent effects.[20] Low membrane permeability of bacitracin is a limitation if its target is to block the oxidase activity of PDI that is catalyzed in the ER. However, the reductive function of PDI is not affected by membrane permeability as it occurs mainly on the cell surface. DTNB and pCMBS react nonspecifically with free thiols and are not specific for PDI. The compounds aPAO and GSAO are blockers of the *CXXC* vicinal cysteines, which are present in many PDI family members as well as other proteins, making them highly toxic.[21]

In this study we employed high-throughput screening (HTS) of RIKEN Natural Product Depository (NPDepo) compounds to identify therapeutically potent PDI inhibitors. We synthesized a series of derivatives of juniferdin, the most potent inhibitor found in NPDepo, and studied the structure−activity relationships of the derivatives. Furthermore, we examined the specificity of the identified PDI inhibitors in terms of their function and homologous protein targets and investigated the potential use of these inhibitors in blocking the PDI-catalyzed reduction of disulfide bonds in the HIV-1 envelope glycoprotein gp120, with a view to using these compounds as inhibitors of HIV-1 entry.

RESULTS AND DISCUSSION

Identification of Juniferdin as a PDI Inhibitor. We sought to identify small molecules that would potently and specifically block the reductase activity of cell surface PDI. We therefore screened the compounds of NPDepo using a high-throughput turbidometric assay system.[22] PDI catalyzes the reduction of insulin in the presence of dithiothreitol (DTT); the reduced insulin chains aggregate, and turbidometry is monitored spectrophotometrically at 620 nm.

We screened about 10,000 NPDepo compounds in a high-throughput manner. After three rounds of screening, we identified four putative PDI inhibitors: estrone, diethylstilbestrol,

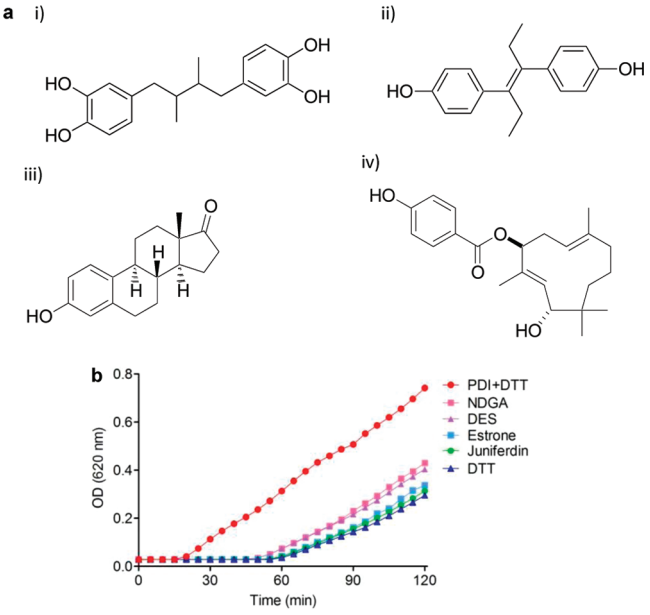
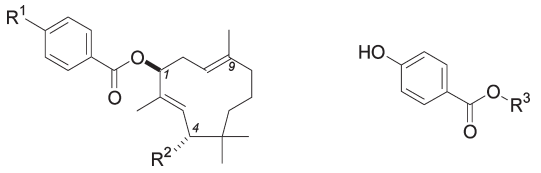


Figure 1. Screening for PDI inhibitors. (a) Structure of the four leading PDI inhibitors: (i) nordihydroguiaretic acid, NDGA; (ii) diethylstilbesterol, DES; (iii) estrone; and (iv) juniferdin. (b) Effect of putative inhibitors on PDI reductase activity. Compounds were assayed at a final concentration of 10 μM, with juniferdin showing the highest inhibitory activity.

nordihydroguiaretic acid, and juniferdin (Figure 1, panel a). Estrone is a naturally occurring estrogen secreted by ovaries during menopause,[23] and diethylstilbestrol is an orally active synthetic nonsteroidal estrogen.[24] Both estrone and diethylstilbesterol were found to inhibit the reduction activity of PDI.[17] Although their mechanism of inhibition is still obscure, PDI contains binding sites for estrogens and can act as a reservoir for hormones.[25] Thus, inhibition of PDI reductase activity by estrogens is likely to occur through the binding of these hormones. The putative PDI inhibitor nordihydroguiaretic acid has been described as a potent antioxidant that may reduce cell damage [26] and inhibit membrane trafficking.[27] Juniferdin, a sesquiterpenoid isolated from the plant *Ferula junipernia*, has been reported to possess estrogenic activity.[28] Hence, we compared the inhibitory potency of juniferdin and previously described estrogenic compounds, 17-β estradiol and estrone, [17] by the insulin reduction assay. Our results suggest both 17-β estradiol and estrone are weaker inhibitors than juniferdin (Supplementary Figure 1, panel a and panel b). Though at higher concentration (10 μM) they show almost similar inhibition, at lower concentrations (1 and 0.1 μM) estrogenic compounds are much less potent. We also checked the effect of juniferdin and 17-β estradiol on MCF-7 proliferation. We found juniferdin has a very low stimulatory effect on MCF-7 proliferation compared to 17-β estradiol (Supplementary Figure 2). Moreover, a comparison of the four PDI hit inhibitors, each at a concentration of 10 μM, showed that juniferdin was the most potent (Figure 1, panel b). We therefore selected juniferdin as the lead PDI inhibitor for further analyses.

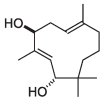
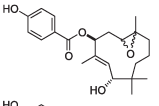
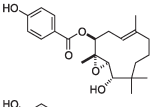
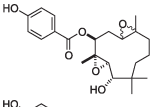
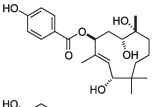
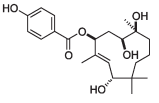
Synthesis and Structure−Activity Relationship of Juniferdin Derivatives. The potent PDI-inhibitory activity of juniferdin prompted us to synthesize its derivatives to generate inhibitors with improved potency and to examine structure−activity relationships (SAR). The general approach we used to synthesize these derivatives is shown in Supplementary

Table 1. Potency (IC_{50}) of PDI Inhibition by Juniferdin and Its Derivatives 2−11



compound	R^1	R^2	R^3	IC_{50} (μM)
1: juniferdin	OH	OH		0.156
2	OMe	OH		>10
3: juniferidin	OH	OAc		0.453
4	OAc	OH		0.627
5	OAc	OAc		0.693
6	H	OH		4.96
7	F	OH		0.958
8			1-octyl	>10
9			cyclooctyl	2.85
10			cyclododecyl	0.565
11			(1*R*)-menthyl	0.667

Table 2. Potency (IC_{50}) of PDI Inhibition by Juniferdin Derivatives 12−17

Compound	Structure	IC_{50} (μM)
12: Juniferol		>10
13		0.167
14		2.534
15		>10
16		>10
17		1.853

Scheme 1. We focused on derivatizing its R^1 (the *para* position of the benzoate), R^2 (the C-4 hydroxyl group of the sesquiterpene ring), and R^3 (the sesquiterpene ring) groups. The structures of these compounds are shown in Supplementary Table 1.

We found that the *p*-methoxybenzoate (**2**) derivative completely abolished the PDI-inhibitory activity of juniferdin, the *p*-fluoro derivative (**7**) reduced its inhibitory activity 6-fold, and the benzoate (**6**) without a *p*-hydroxyl group reduced its activity 30-fold (Table 1). In contrast, the monoacetates (**3**, **4**) and the diacetate (**5**) showed moderate activity (3- to 4-fold less than **1**). In investigating the importance of the sesquiterpene ring, we found that the octyl 4-hydroxybenzoate (**8**) had no inhibitory activity, whereas the inhibitory activity of the cyclooctyl derivative (**9**) was 18-fold lower than that of **1**. Interestingly, derivatives with substituted cyclododecyl (**10**) and (1*R*)-menthyl (**11**) groups showed good inhibitory activity.

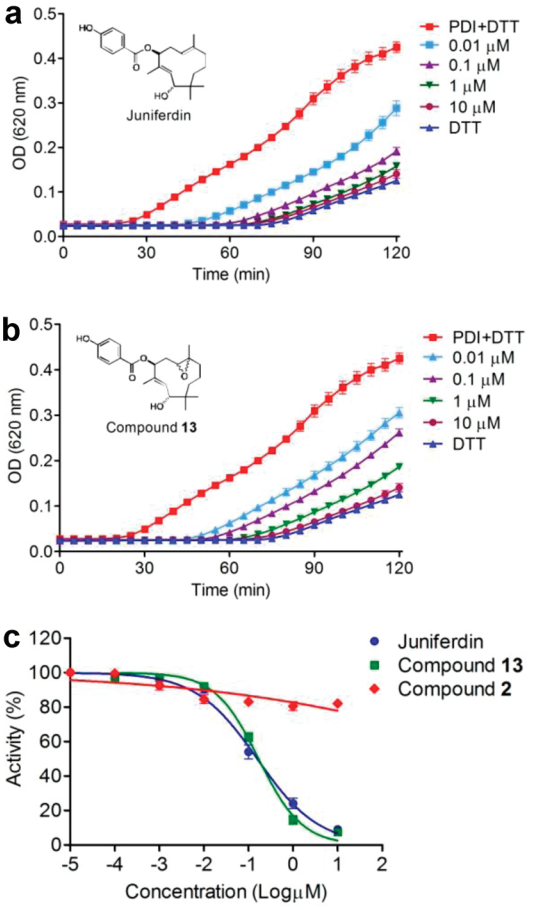


Figure 2. Dose-dependent inhibition of PDI by (a) juniferdin and (b) compound **13**. PDI reductive activity was measured in the presence or absence of 10, 1, 0.1, or 0.01 μM juniferdin or compound **13** in a 100-μL reaction volume containing 1.3 μM PDI, 0.5 mM DTT, 0.13 mM insulin, 100 mM sodium phosphate buffer, 2 mM EDTA. (c) IC_{50} curves for juniferdin, compound **13**, and compound **2**. The IC_{50} values of juniferdin and compound **13** were 156 and 167 nM, respectively.

The most promising results came from SAR investigations of compounds with a modified sesquiterpene ring (Table 2). Juniferol, a dihydroxy derivative with a sesquiterpene 11-membered ring system, showed no inhibitory activity. The 9,10-monoepoxide (**13**) 1:1 stereoisomers showed inhibition similar to that of **1**, whereas the 2,3-monoepoxide (**14**) showed 15-fold lower inhibition, and the 2,3,9,10-diepoxide (**15**) was detrimental to activity. Of the triols in the sesquiterpene ring, the 4,9,10-trihydroxy derivative (**16**) showed no inhibition, whereas the stereoisomeric 4,9,10-trihydroxy derivative (**17**) showed modest inhibition. Thus these SAR studies revealed that both benzoate moiety and cyclic alcohol were indispensable and a *p*-hydroxybenzoate group was important for strong inhibition of PDI activity. It was also interesting that the hydroxyl phenyl group was present on all four putative inhibitors of PDI (Figure 1, panel a). In summary, compound **13** was the most potent inhibitor of the derivatives we synthesized, whereas compound **2** showed the least inhibition.

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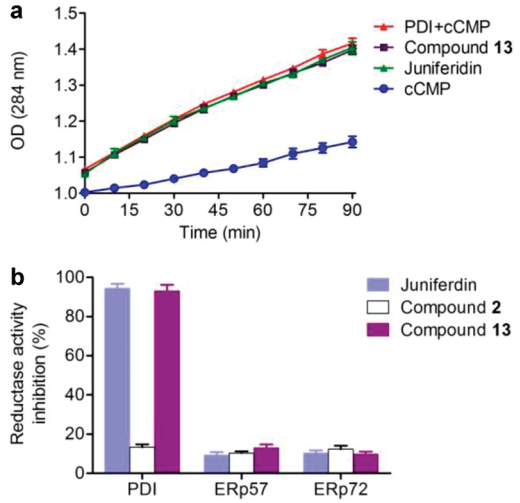

Figure 3. Specificity of juniferdin, compound **2**, and compound **13**. Effects of 10 μM juniferdin, compound **2**, and compound **13** on (a) PDI oxidase activity, measured by the continuous RNase refolding assay, and (b) PDI, ERp57, and ERp72 reductase activity, measured by the insulin turbidometric assay method.

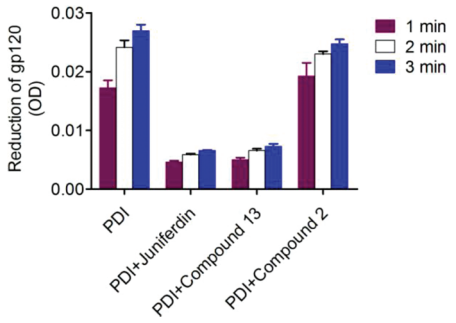

Figure 4. Inhibition of PDI-mediated reduction of HIV-1_{III-B} gp120 by juniferdin and compound **13**. ELISA plates were coated with recombinant gp120 and incubated for 1−3 min with PBST, 0.35 μM PDI, 0.5 mM DTT, and 1 μM compounds. Free-SH groups were labeled by NEM-biotin and exposed to streptavidine-ALP. All reactions were normalized relative to DTT at each time point.

Dose-Dependent Inhibition of PDI Activity by Juniferdin and Compound 13. When we assayed the dose-dependency of juniferdin on PDI reductase activity at 10, 1, 0.1, or 0.01 μM, using the insulin turbidometry assay, we found that juniferdin inhibited PDI reductase dose-dependently (Figure 2, panel a) and at 10 μM, juniferdin inhibited approximately 98% of the PDI reductase. In addition, compound **13** showed dose-dependent inhibition of PDI (Figure 2, panel b). Dose−response curves showed that the IC_{50} values for juniferdin and compound **13** were 156 and 167 nM, respectively (Figure 2, panel c).

To date, juniferdin is the most potent inhibitor of PDI reduction activity. Of the previously described PDI inhibitors, bacitracin has been found to inhibit ∼95% PDI activity at nonphysiological concentrations (3 mM), whereas the most potent estrogen (17-β estradiol) showed 40−60% inhibition at 1 μM, and membrane-impermeable sulfhydryl blockers DTNB and pCMBS completely inhibited PDI at 1 mM.[5,15,17] Since juniferdin and compound **13** exerted ∼98% PDI inhibition at 10 μM concentrations, they are 10- to 300-fold more potent than the previously mentioned inhibitors (Supplementary Table 2). Moreover, juniferdin and compound **13** inhibited only PDI, whereas bacitracin, DTNB, and pCMBS inhibited many sulfhydryl-group-containing enzymes.

Specific Inhibition of PDI Reductase Activity by Juniferdin and Compound 13. To confirm the functional specificity of juniferdin and compound **13**, we examined their effects on the oxidase activity of PDI. The oxidase activity was monitored by measuring the continuous refolding of denatured RNase.[29] PDI catalyzes the oxidative renaturation of reduced, denatured RNase in a glutathione redox buffer. The oxidized RNase hydrolyzes the substrate cCMP to CMP, which can be measured at 284 nm. We found that, at final concentrations of 10 μM, neither juniferdin nor compound **13** had an effect on the oxidative folding activity of PDI (Figure 3, panel a). We also measured the PDI-mediated oxidation of reduced lysozyme. According to this assay, PDI reactivates lysozyme in a glutathione redox buffer and in turn lysozyme clears the suspension of *Micrococcus lysodeikticus* cell wall. We found that juniferdin neither at 1 nor 10 μM showed significant inhibition of PDI oxidase activity (Supplementary Figure 3), thus confirming the reaction specificity.

To investigate the target specificity of juniferdin and compound **13**, they were tested against proteins similar to PDI in structure and activity. PDI is a 17-member protein family that belongs to the thioredoxin superfamily. In the PDI family, ERp57 and ERp72 show reductase activity similar to that of PDI and share the same active site sequence, CGHC. Although PDI and ERp57 each have two active sites, ERp72 contains three active sites.[30] We investigated whether juniferdin and compound **13** were specific inhibitors of PDI or if they could also inhibit the reduction activity of ERp57 and ERp72, as determined using insulin turbidometry assays. We found that, at 10 μM, both juniferdin and compound **13** showed negligible inhibition (∼10%) of ERp57 and ERp72 reduction activity (Figure 3, panel b). Furthermore these PDI members contain thioredoxin-like domains; we therefore evaluated whether juniferdin could inhibit thioredoxin mediated insulin reduction. We found that juniferdin did not inhibit thioredoxin reductase activity (Supplementary Figure 4). In conclusion our results show that juniferdin and compound **13** specifically inhibit the reduction activity of PDI.

PDI enzymatic activity depends on domain architecture and the presence or absence of additional residues that may regulate the pK_a of the active site cysteines. PDI consists of four domains, **a**, **b**, **b**′, and **a**′, with a linker region between **b**′ and **a**′ and a C-terminal acidic region (Supplementary Figure 5). The **a** and **a**′ domains are catalytically active, while the **b**′ domain functions as a peptide binding domain. Either **a** or **a**′ is required for oxidase activity, and the linear combination of one of these with the substrate binding domain **b**′ is required for simple isomerization.[9] In contrast, all four of these domains are needed for complex isomerization, causing major conformational changes, which can be achieved through cycles of reduction and oxidation. Moreover, the residues that influence the pK_a values of the active site cysteines also regulate PDI functions. The exact reasons behind the specificity of juniferdin and compound **13** to PDI reductase activity are still obscure. Presumably these inhibitors bind to specific residues or domains that are required only for PDI reductase activity.

Juniferdin and Compound 13 Inhibit the PDI-Catalyzed Reduction of gp120. Since the PDI-catalyzed reduction of disulfides in gp120 has been shown to be crucial for HIV-1 entry into cells,[10,11,15] we examined whether juniferdin and compound **13** could inhibit the PDI-catalyzed reduction of gp120. HIV-1 gp120 was fixed onto ELISA plates, which were incubated with

the potential inhibitor and DTT in the presence or absence of PDI for a fixed time, and NEM-biotin was used to specifically react with the reduced thiols produced by the PDI. Increased time-dependent formation of free thiols by PDI was observed, in accordance with published results.[31] However, we found that both juniferdin and compound **13** significantly inhibited the PDI-catalyzed reduction of gp120, while compound **2** showed no inhibition (Figure 4). In conclusion, juniferdin and compound **13** inhibit the PDI-catalyzed reduction of HIV gp120, showing that they may inhibit viral entry.

Several anti-PDI agents, such as DTNB, bacitracin, PAO, and anti-PDI antibodies, have been used to inhibit the PDI-mediated reduction of gp120 and subsequent HIV infection. DTNB, bacitracin, and anti-PDI antibody have been found to markedly inhibit HIV infection of human lymphocytes,[15] as have aPAO and AT3.[10] Although initially showing promising results, these agents could not be used therapeutically due to their nonspecific activities and toxicities. The two compounds we identified, juniferdin and compound **13**, showed specific inhibition of PDI reductase activity and did not inhibit the other homologues of PDI. Hence, their ability to inhibit the PDI-catalyzed reduction of HIV gp120 suggests that juniferdin and compound **13** may be promising as inhibitors of viral entry.

Since these compounds showed promising therapeutic value, we investigated whether they possess any cytotoxic activity. Using the WST-8 assay, we measured the effects of juniferdin and compound **13** on the viability of HeLa, HepG2, HL-60, HT1080, and K562 cell lines. We found that compound **13** was less cytotoxic than juniferdin against all cell lines (Supplementary Table 3).

Summary and Implications. Using HTS we identified juniferdin as a novel small molecule PDI inhibitor. Using juniferdin as a lead compound, we synthesized a series of derivatives, with SAR assays showing that compound **13** had inhibitory activity similar to that of juniferdin. Both inhibitors specifically blocked the reductase activity of PDI, a selective feature that can be therapeutically harnessed in situations where reduction-dependent protein conformational changes occur on the cell surface. For example, PDI-catalyzed reduction of disulfide bonds in gp120 is a prerequisite for HIV-1 entry into host cells (Supplementary Figure 6). We found that the PDI inhibitors juniferdin and compound **13** inhibited the PDI-catalyzed reduction of the disulfide bonds in gp120. These specific PDI inhibitors may be promising antiviral therapeutic agents. Moreover, by inhibiting viral entry into cells, these inhibitors may act at the starting point of a disease cycle, thereby quenching disease progression at the earliest possible stages. Future confirmation of the antiviral properties *in vivo* of these compounds may provide a solid basis for their therapeutic development.

The promiscuity of PDI and its activity in reducing disulfide bonds make this enzyme a prime target for therapeutic agents. Reduction of disulfide bonds by surface-associated PDI is important for the entry of virus and toxins into the cells. For example, the cholera and diphtheria toxins contain disulfide bonds cleaved by PDI, thus facilitating the passage of the toxins. Functionally specific PDI inhibitors such as juniferdin and compound **13** may therefore largely prevent the maturation of toxins that are dependent on disulfide bond cleavage. Thus, selective inhibitors of PDI are likely to find use in a broad range of therapeutic applications.

METHODS

Construction of Plasmids and Establishment of Stable Cell Lines. The ER retention signal sequence (KDEL)-deleted human PDI cDNA was subcloned into the pcDNA3.1/Myc-His (+) vector (Invitrogen). A stable cell line expressing PDI was established by transfecting pcDNA3.1-PDI into HT1080 cells using Effectence Transfection Reagent (QIAGEN), followed by selection with 400 μg/mL G418. Cells transfected with pcDNA3.1-PDI were designated HT1080-PDI-MH cells. Human ERp57 and ERp72 cDNA [32] without ER retention sequence (QEDL and KEEL, respectively) were subcloned into the pcDNA3.1/Myc-His (+) vector. Stable cell lines prepared by transfecting HT1080 cells with clones plasmids were designated HT1080-ERp57-MH and HT1080-ERp72-MH cells, respectively.

Purification of PDI, ERp57, and ERp72. Recombinant PDI, ERp57, and ERp72 were purified as described previously [33] with slight modifications. Briefly, cells overexpressing PDI, ERp57, or ERp72 were grown to confluence, washed three times with serum-free DMEM, and incubated in serum-free media for 24 h. The conditioned media were collected and incubated with Ni-NTA agarose (Qiagen) for 2 h at 4 °C. The Ni-NTA agarose was washed thrice with sodium phosphate buffer, and Ni-NTA agarose-bound PDI, ERp57, or ERp72 was eluted with 500 mmol/L imidazole in sodium phosphate buffer and electrophoresed on SDS-polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue G-250. Western blotting with anti-Myc (sc-40, Santa Cruz Biotechnology) antibody was performed as described.[33]

Measurement of PDI Reductase Activity. Reductase activity was assayed by measuring the PDI-catalyzed reduction of insulin in the presence of DTT, thus measuring the aggregation of reduced insulin chains at 650 nm.[34] The incubation mixture contained 100 mM sodium phosphate buffer pH 7.0, 0.5 mM DTT, 2 mM EDTA, 0.13 mM bovine insulin (Sigma), and 1.3 μM purified human PDI.

High-Throughput Screening for PDI Inhibitors. A high-throughput screening (HTS) system was used to search for PDI inhibitors. For HTS, 8664 compounds at four concentrations each, 3.33, 1.111, 0.33, and 0.11 mg/mL, were collected from NPDepo, RIKEN [35] in 96-well plates. To each well was added a reaction mixture containing 2 mM EDTA, 0.13 mM insulin, 10 μM compounds, 1.3 μM PD,I and 0.5 mM DTT in 100 mM sodium phosphate buffer, using a Biomek 2000 liquid handling system (Beckman). After about 30 min, absorption was measured spectrophotometrically at 650 nm (Wallac 1420 ARVO, PerkinElmer), for 2 h at 5 min intervals. Following the first step of HTS, using a robotic system, 201 compounds were selected as primary hits. The second step was performed manually to minimize machine error and artifacts. In the first and second steps, compounds were assayed at 0.33 mg/mL. After the second step 24 compounds were selected for the next round. In the third step compounds of different concentrations were used, and finally four putative PDI inhibitors were identified.

Measurement of PDI Oxidase Activity. Reduced and denatured RNase A was prepared as described.[36] Ten milligrams of native bovine RNase A (Sigma) was incubated in 2 mL of 6 M guanidine HCl in 0.1 M Tris-acetate (pH8.0), 2 mM EDTA, and 0.15 M DTT. The reduced and denatured RNase A was separated from DTT and guanidine-HCl using Sephadex G-25 equilibrated with 0.1% (v/v) acetic acid. PDI oxidase activity was measured using a continuous RNaseA refolding assay,[29,37] in which PDI catalyzes the oxidative renaturation of RNase A in a glutathione redox buffer, and the oxidized RNase A then hydrolyzes the substrate cytidine 2′-3′-cyclin monophosphate (cCMP), monitored spectrophotometrically at 284 nm. Typical assays contained 100 mM sodium phosphate buffer, pH 7.5, 2 mM GSH, 0.2 mM GSSG, 8 μM reduced RNaseA, 1.4 μM PDI, and 4.5 mM cCMP. The reaction mixture was equilibrated at 25 °C for 15 min before adding the substrate cCMP. Absorbance was measured at 284 nm for 1 h at 5 min intervals.

Synthesis of Juniferdin Derivatives. All compounds were prepared using the procedure shown in Supplementary Scheme 1. All moisture-sensitive reactions were performed under a nitrogen atmosphere with dehydrated solvents under anhydrous conditions. Juniferdin

was purchased from Namiki Shoji Co. Ltd. All reagents and solvents were purchased from commercial suppliers and used without further purification. All reactions were monitored by thin-layer chromatography (TLC) on 0.25 mm Merck silica gel plates (60 F_{254}) using UV light, a color reaction with 10% ethanolic phosphomolybdic acid, and heat as detecting methods. All compounds were purified by preparative TLC using 0.5 mm Merck silica gel plates (60 F_{254}) or by HPLC using Sensyu Pak PEGASIL Silica 120-5 (200 mm × 250 mm) (Sensyu Scientific Co., Ltd.). The structure and purity of all compounds were confirmed by ^{1}H NMR spectra recorded on a JEOL JNM-ECP-500 MHz spectrometer in $CDCl_3$. Treatment of juniferdin (**1**) with acetic anhydride-pyridine in CH_2Cl_2 at RT resulted in the monoacetate **4** as the major product, along with the monoacetate **3** (juniferidin) and diacetate **5**. The *p*-methoxy benzoate **2** was synthesized by methylation of **1** with trimethylsilyldiazomethane ($TMSCHN_2$). Juniferdin was hydrolyzed with 1 N NaOH in MeOH at 60 °C to yield juniferol **12**. Oxidation of **1** with *m*-chloroperbenzoic acid (MCPBA) yielded the 9,10-monoepoxide **13** (1:1 stereoisomers) and the diepoxide **15** (4:1 stereoisomers), whereas oxidation of **1** with *tert*-butylhydroperoxide in the presence of vanadyl acetyacetonate yielded the 2,3-monoepoxide **14** (single stereoisomer) and the diepoxide **15** (4:1 stereoisomers). Oxidation of **1** with osmium tetroxide yielded the triol **16** as the major product together with a small amount of the stereoisomer **17**. Benzoate **6** and *p*-fluorobenzoate **7** were prepared by esterification of jufiferol **12** with the corresponding acid chloride, and the *p*-hydroxybenzoates **8**−**11** were prepared by esterification of *p*-hydroxybenzoic acid with the corresponding alcohol and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDCI). The structure of each compound was confirmed by examination of spectra data. The spectra data have been included in Supporting Information.

Inhibition of PDI-Mediated Reduction of HIV-1 gp120. PDI-mediated reduction of HIV-1_{IIIB} gp120 was assayed as described, with slight modifications.[31] Recombinant HIV-1_{IIIB} gp120 produced in CHO cells was purchased from ImmunoDiagnostics, and 96-well MaxiSorp ELISA plates (Nunc) were coated with 4 μg/mL gp120 diluted in carbonate buffer, pH 9.6 at 4 °C overnight. The plates were washed three times with PBST (phosphate -buffered saline, pH 7.4, plus 0.05% Tween-20) and the PDI-mediated reduction of gp120 was determined by incubating the wells with PBST in the presence or absence of 0.35 μM PDI, 0.5 mM DTT, and 10 μM compounds for 1−3 min. The plates were washed four times with PBST and incubated for 1 h at RT with 2 μM NEM-Biotin (Sigma), which reacts specifically with thiol groups. The wells were again washed four times with PBST and incubated for 1 h at RT with alkaline phosphatase conjugated streptavidine (MABTECH). After 4 washes with PBST, the plates were incubated in the dark for 15 min with *p*-nitrophenyl phosphate (Sigma), and absorbance was measured at 405 nm using Wallac 1420 ARVO.

Statistical Analysis. PDI activity in the presence of compound was determined by the formula, PDI activity (%) = $(OD_{[PDI+DTT+compound]} - OD_{DTT})/(OD_{[PDI+DTT]} - OD_{DTT}) \times 100$. OD values were taken from the exponential phase (95 min) of the reaction. For the calculation of IC_{50} values, compounds from 10 μM to 0.1 nM were used. The IC_{50} curves were analyzed by using the equation "log(inhibitor) vs normalized response-variable slope" of nonlinear regression to determine the IC_{50} values. The errors bars indicate mean ± SE of three replicates. All graphs were drawn using GraphPad Prism 5 software.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 81-48-467-9542. Fax: 81-48-462-4669. E-mail: hisyo@riken.jp.

Present Addresses

¶Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia.

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■ ABBREVIATIONS USED

PDI,protein disulfide isomerase; HIV,human immunodeficiency virus; gp,glycoprotein; DTNB,5,5′-dithiobis(2-nitorbenzoic acid); PAO,phenylarsine oxide; aPAO,*p*-amino PAO; GSAO, 4-(*N*-(*S*-glutathionylacetyl)aminophenylarsenoxide; pCMBS, *p*-chloromercuriphenylsulfonate

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