

Development of a hematopoietic prostaglandin D synthase-degradation inducer

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Abstract

Although hematopoietic prostaglandin D synthase (H-PGDS) is an attractive target for treatment of a variety of diseases, including allergic diseases and Duchenne muscular dystrophy, no H-PGDS inhibitors have yet been approved for treatment of these diseases. Therefore, the development of novel agents having other mode of actions to modulate the activity of H-PGDS is required. In this study, a chimeric small molecule that degrades H-PGDS via the ubiquitin-proteasome system, **PROTAC(H-PGDS)-1**, was developed. **PROTAC(H-PGDS)-1** is composed of two ligands, TFC-007 (that binds to H-PGDS) and pomalidomide (that binds to cereblon). **PROTAC(H-PGDS)-1** showed potent activity in the degradation of H-PGDS protein via the ubiquitin-proteasome system and in the suppression of prostaglandin D₂ (PGD₂) production. Notably, **PROTAC(H-PGDS)-1** was slightly more effective in the suppression of PGD₂ production than the known inhibitor, TFC-007. Thus, the H-PGDS degrader—**PROTAC(H-PGDS)-1**—is expected to be useful in biological research and clinical therapies. (145 words)

Overproduction of PGD₂ is related to a variety of diseases, including allergic diseases,^{1,2} physiological sleep,³ and Duchenne muscular dystrophy.⁴ H-PGDS is one of the enzymes involved in PGD₂ synthesis; therefore, H-PGDS is a potential therapeutic target for such diseases, for example, in the nasal mucosa of patients with allergic rhinitis.⁵ *In vivo* studies have demonstrated that H-PGDS inhibition is effective in the treatment of allergic inflammation.⁶⁻⁸ To date, several types of H-PGDS inhibitors have been developed as therapies for allergic and inflammatory responses. However, the advancement of these inhibitors into clinical studies has not been satisfactory. Thus, the development of novel agents for clinical investigation with modes of action other than H-PGDS inhibition is required.

In recent years, innovative chimeric drugs, PROTACs (proteolysis targeting chimeras) and SNIPERs (specific and non-genetic inhibitor of apoptosis protein [IAP]-dependent protein erasers), which enable the degradation of target proteins via the ubiquitin-proteasome system (UPS), have been developed. These drugs are chimeric molecules, composed of target protein ligands and E3 ligase ligands, which recruit a target protein in proximity to an E3 ligase to induce protein degradation.⁹⁻¹¹ Some degraders have been reported to show more durable suppression of the cellular responses caused by the target protein than small molecule inhibitors.¹²⁻¹⁴ A variety of PROTACs and SNIPERs have been developed for the treatment of cancer that targeted related proteins, such as transcriptional regulators, nuclear receptors, and protein kinases.^{11,15,16} As a new mechanism of action for regulating the activity of H-PGDS, the development of degraders targeting the H-PGDS protein is an attractive approach for the treatment of chronic allergic diseases. In the present study, we developed the chimeric small molecule **PROTAC(H-PGDS)-1**, which had potent activity for the

degradation of H-PGDS protein via the UPS and in the suppression of PGD₂ production.

Results and Discussion

Among the representative H-PGDS selective inhibitors, HQL-79,¹⁶ F092,¹⁸ BSPT,¹⁹ TAS-204,⁷ and TFC-007, TAS-204, and TFC-007 show high activity (with IC₅₀ values of 23 and 83 nM, respectively, Figure 1a).⁵⁻⁸ The binding mode between H-PGDS and F092 (and HQL-79) has been revealed by X-ray diffraction.^{17,18} From the above information, we focused on the chemical structures of F092 and TFC-007, which contain the same *N*-phenyl-5-pyrimidinecarboxamide moiety, to design a chimeric molecule. In the X-ray crystal structure of the ligand binding domain of H-PGDS and F092 (PDB: 5YWX), the *N*-phenyl-5-pyrimidinecarboxamide moiety faces to the inside and the 2-pyrrolidone moiety is orientated to the outside of the protein (Figure 1b). We hypothesized that the elongated structure of TFC-007, similar to F092, would be suitable as a target protein ligand and the morpholine moiety of TFC-007 would face the surface of the protein. Thus, we designed a TFC-007-based PROTAC by the replacement of the morpholine moiety with a piperazine moiety for linking with the E3 ligase ligand. As the E3 ligase ligand, pomalidomide was selected, which binds to cereblon (CRBN). CRBN is a broadly expressed protein and forms part of the cullin-4-containing E3 ubiquitin ligase complex (CRL4). The structures of **PROTAC(H-PGDS)-1** and the negative control **PROTAC(H-PGDS)-2** with *N*-methylated pomalidomide, which is considered to have considerably reduced binding affinity to CRBN,¹² are shown in Figure 1c. The synthetic routes for

PROTAC(H-PGDS)-1 and **PROTAC(H-PGDS)-2** are given in the supporting information (Schemes S1 and S2, respectively).

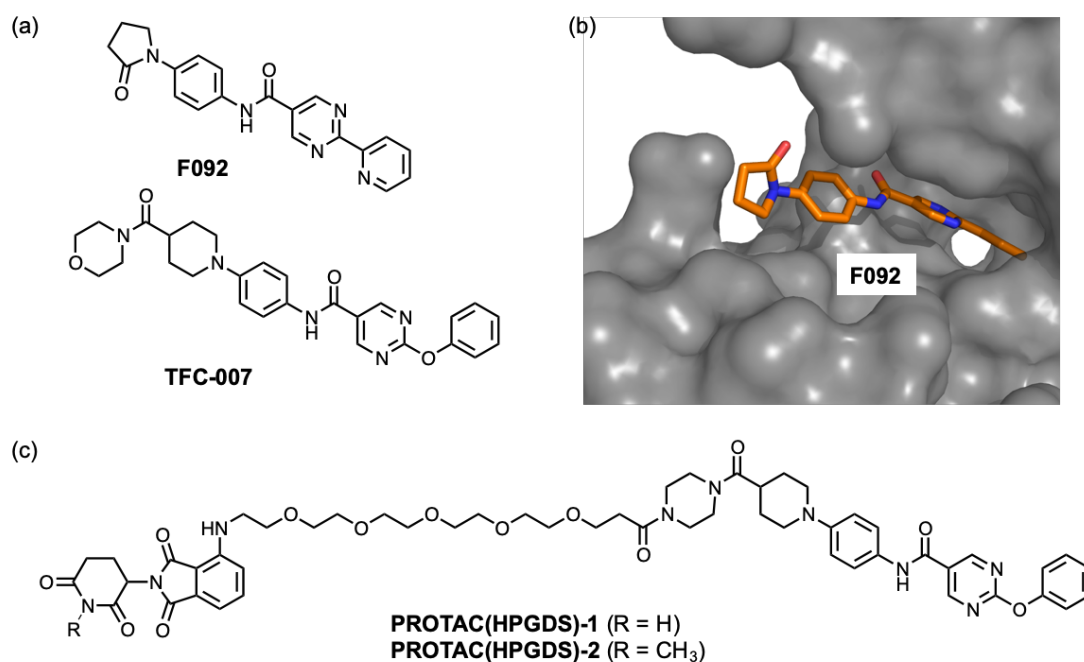


Figure 1. (a) Chemical structures of the H-PGDS inhibitors F092 and TFC-007. (b) X-ray crystal structure of H-PGDS with F092 (PDB: 5YWX). (c) Chemical structures of **PROTAC(H-PGDS)-1** and **PROTAC(H-PGDS)-2**.

To examine the effect of **PROTAC(H-PGDS)-1** on H-PGDS protein levels, human KU812 cells expressing H-PGDS protein were treated with graded concentrations of **PROTAC(H-PGDS)-1** for 3 h (Figure 2a). Effective reduction of H-PGDS protein by **PROTAC(H-PGDS)-1** was observed at concentrations ≥ 10 nM and the maximum activity was observed at 100–1000 nM. Additionally,

PROTAC(H-PGDS)-1, at concentrations ≥ 10 nM, showed more potent activity in the reduction of the H-PGDS protein when the cells were incubated with **PROTAC(H-PGDS)-1** for 6 or 24 h (Figure 2a). Similar protein reduction activity for **PROTAC(H-PGDS)-1** was also observed in MEG-01s cells expressing H-PGDS protein (Figure S1). This reduction activity was not observed for **SNIPER(H-PGDS)-1**, in which TFC-007 is conjugated to an IAP ligand LCL161 derivative (Figure S2). While **PROTAC(H-PGDS)-1** showed potent activity in H-PGDS protein reduction, **PROTAC(H-PGDS)-1** had little effect on the protein levels of mPGES-1, one of the major enzymes for PGE₂ synthesis (Figure S3),²⁰ suggesting that **PROTAC(H-PGDS)-1** may specifically reduce H-PGDS and not other prostaglandin (PG) synthases.

To understand the mechanism for the reduction of H-PGDS protein by **PROTAC(H-PGDS)-1**, we examined the turnover of H-PGDS protein after **PROTAC(H-PGDS)-1** treatment. When KU812 cells were treated with the protein synthesis inhibitor cycloheximide in KU812 cells, the levels of H-PGDS protein were dramatically decreased within 6 h in the **PROTAC(H-PGDS)-1**-treated cells, but were retained in the control cells (Figure 2b). In contrast, the turnover rate of cyclin B1 was not affected by **PROTAC(H-PGDS)-1** treatment. These results indicated that **PROTAC(H-PGDS)-1** selectively induces a reduction in H-PGDS protein levels. Furthermore, the levels of *H-PGDS* mRNA in KU812 cells were not affected by **PROTAC(H-PGDS)-1** (Figure 2c). These results indicated that **PROTAC(H-PGDS)-1** induces the degradation of H-PGDS protein.

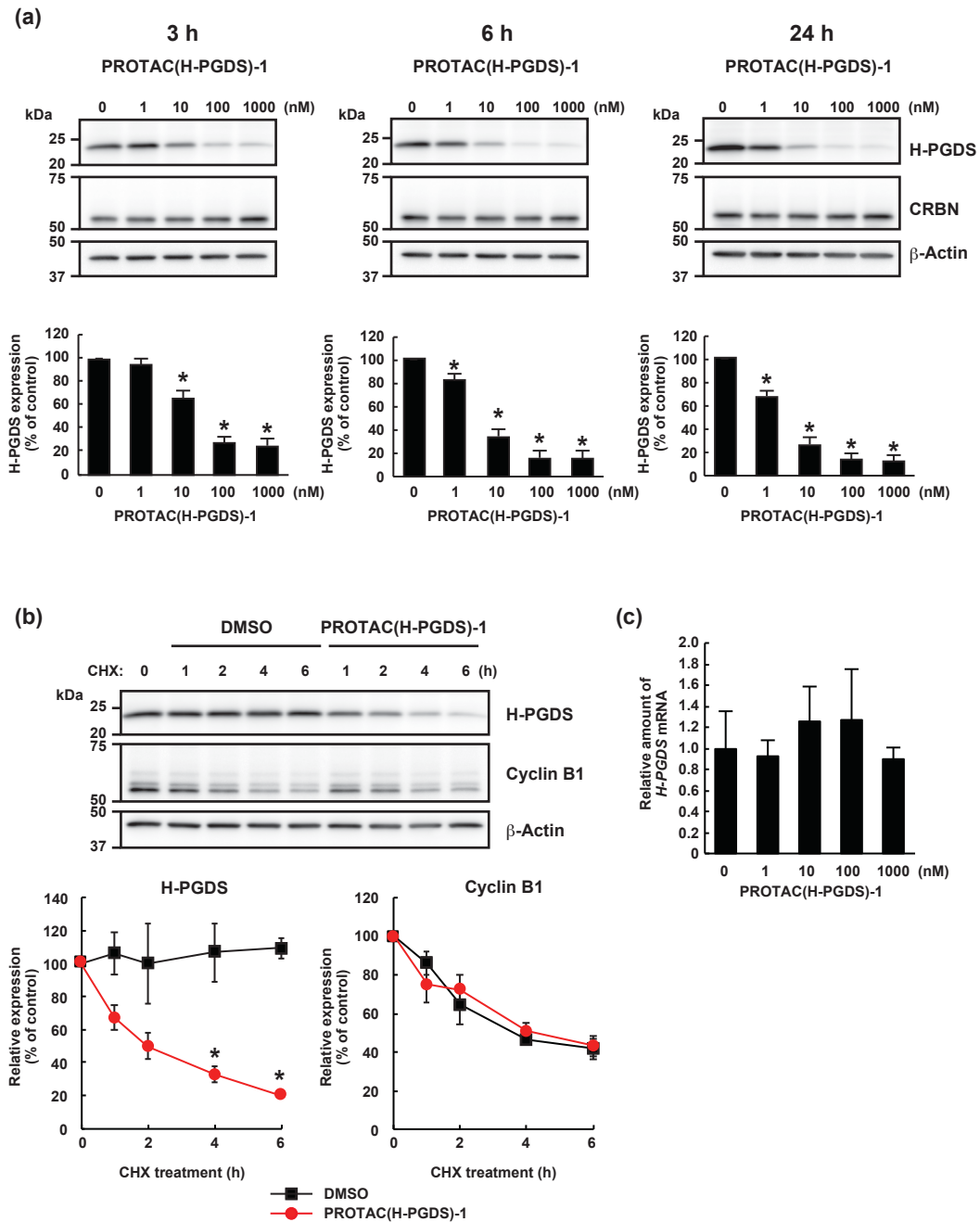


Figure 2. PROTAC(H-PGDS)-1 is a degrader for H-PGDS protein. (a) KU812 cells were incubated with the indicated concentration of **PROTAC(H-PGDS)-1** for the indicated time. The H-PGDS/ β -Actin ratio was normalized by the vehicle control as 100. Data in the bar graph are the mean \pm SD ($n = 3$). (b) Turnover of H-PGDS protein in KU812 cells after treatment with 10 μ g/ml of cycloheximide (CHX) in the presence or

absence of 100 nM of **PROTAC(H-PGDS)-1** for the indicated periods. The H-PGDS/ β -Actin and cyclin B1/ β -Actin ratios were normalized by the time 0 control as 100. Data in the graphs are the mean \pm SD ($n = 3$). (c) Expression of *H-PGDS* mRNA in KU812 cells. Cells were incubated with the indicated concentration of **PROTAC(H-PGDS)-1** for 6 h. Expression levels are relative to vehicle treatment, which was arbitrarily set to 1. Data in the bar graph are the mean \pm SD ($n = 3$). $*P < 0.01$ compared with vehicle-treated control in a two-tailed Student's t test.

To explore the mechanism of **PROTAC(H-PGDS)-1**-induced degradation of the H-PGDS protein, we first examined the effect of TFC-007 and pomalidomide on the H-PGDS protein. While addition of **PROTAC(H-PGDS)-1** resulted in dramatic degradation of H-PGDS protein, the combination of TFC-007 and pomalidomide (1 μ M each) did not effectively decrease the amount of H-PGDS protein (Figure 3a), indicating that linking the two ligands into a single molecule is critically important for the degradation of the H-PGDS protein. **PROTAC(H-PGDS)-1** was designed to recruit CRBN for the degradation of H-PGDS protein. A competition assay using an excess amount of pomalidomide diminished the protein degradation activity of **PROTAC(H-PGDS)-1** (Figure 3b), indicating that CRBN binding was required for the protein degradation.

To investigate the involvement of the UPS in the degradation of H-PGDS protein by **PROTAC(H-PGDS)-1**, we used the proteasome inhibitor MG132 and the ubiquitin-activating enzyme inhibitor MLN7243. The **PROTAC(H-PGDS)-1**-induced degradation of H-PGDS protein was suppressed by the inhibitors (Figure 3c), suggesting that the degradation of H-PGDS protein requires the UPS.

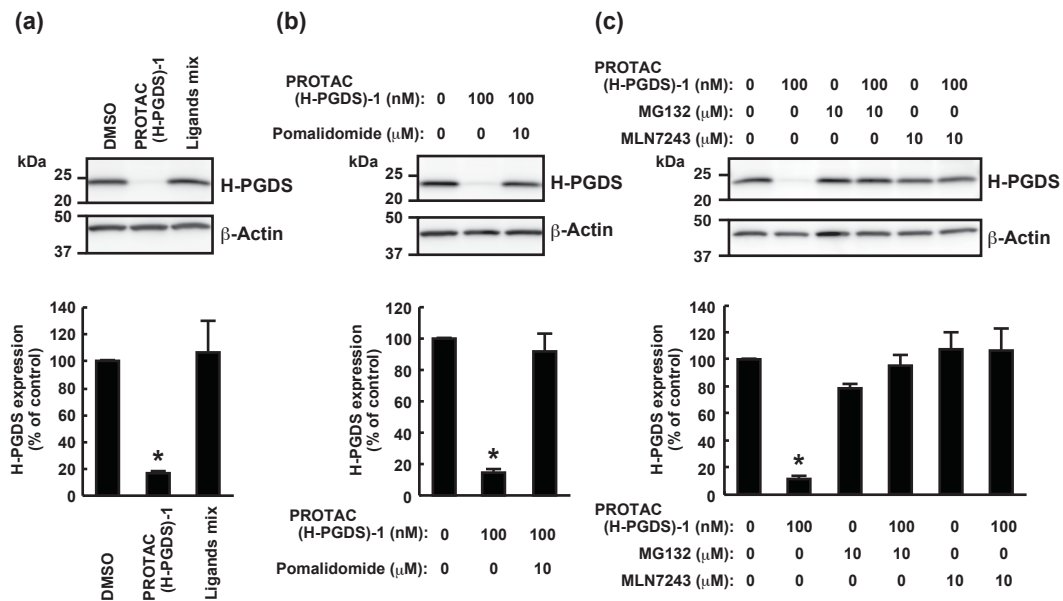


Figure 3. Involvement of the ubiquitin-proteasome system in the **PROTAC(H-PGDS)-1**-induced degradation of H-PGDS protein. (a) KU812 cells were incubated with 1 μ M **PROTAC(H-PGDS)-1** or the ligand mixture (TFC-007 and pomalidomide, 1 μ M each) for 6 h. (b) Competition assay using an excess amount of pomalidomide with **PROTAC(H-PGDS)-1** in KU812 cells. Cells were incubated with 100 nM **PROTAC(H-PGDS)-1** and/or 10 μ M pomalidomide for 6 h. (c) Effect of MG132 and MLN7243 on the protein knockdown activity of **PROTAC(H-PGDS)-1** in KU812 cells. Cells were incubated with 100 nM **PROTAC(H-PGDS)-1** in the presence or absence of 10 μ M MG132 or 10 μ M MLN7243 for 6 h. The H-PGDS/ β -Actin ratio was normalized by the vehicle control as 100. Data in the bar graphs are the mean \pm SD ($n = 3$). * $P < 0.01$ compared to vehicle-treated control in a two-tailed Student's t test.

As discussed above, **PROTAC(H-PGDS)-1** is a potent H-PGDS protein degrader that is dependent on the UPS. In addition to the degradation, **PROTAC(H-PGDS)-1** may also inhibit the enzymatic activity of H-PGDS; this is because it contains the TFC-007 moiety, which inhibits H-PGDS enzymatic activity. To investigate the importance of H-PGDS degradation for the suppression of PGD₂ production, we developed a structurally related inactive degrader as a control compound. **PROTAC(H-PGDS)-2** is composed of TFC-007 and *N*-methylated pomalidomide, which is unable to recruit CRBN.¹² Using fluorescence polarization assays, we investigated the binding affinity to H-PGDS. TFC-007, **PROTAC(H-PGDS)-1**, and **PROTAC(H-PGDS)-2** showed similar affinity toward H-PGDS with IC₅₀ values of 0.32, 0.32, and 0.30 μM, respectively (Figure 4a). We also investigated the inhibitory activity of the compounds against the H-PGDS enzyme *in vitro*. **PROTAC(H-PGDS)-1** and **PROTAC(H-PGDS)-2** had slightly lower IC₅₀ values, 266 nM for **PROTAC(H-PGDS)-1** and 320 nM for **PROTAC(H-PGDS)-2**, than TFC-007 (71 nM, Figure S4). Then, we examined the degradation activity of the compounds against H-PGDS protein. KU812 cells were treated with **PROTAC(H-PGDS)-1**, **PROTAC(H-PGDS)-2**, or TFC-007 for 24 h. **PROTAC(H-PGDS)-1** reduced H-PGDS protein levels, while neither **PROTAC(H-PGDS)-2** nor TFC-007 affected H-PGDS protein levels (Figure 4b). Taken together, these results indicated that **PROTAC(H-PGDS)-1** was a potent degrader and inhibitor of H-PGDS, while **PROTAC(H-PGDS)-2** and TFC-007 inhibited enzymatic activity but did not degrade H-PGDS. Finally, we investigated the effect of **PROTAC(H-PGDS)-1**, **PROTAC(H-PGDS)-2**, and TFC-007 on the production of PGD₂ in KU812 cells. Treatment with the compounds for 24 h suppressed the production of PGD₂, and

PROTAC(H-PGDS)-1 reduced PGD₂ production more effectively than the other compounds (Figure 4c).

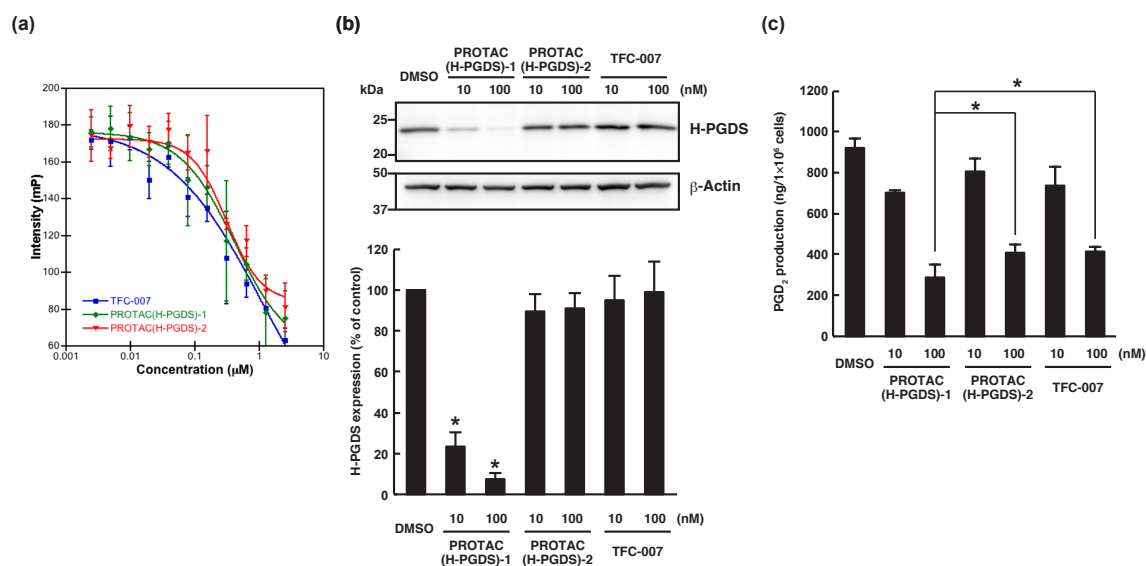


Figure 4. Comparison of the degradation and inhibition of H-PGDS. (a) Fluorescence polarization assays of the binding affinity between H-PGDS and TFC-007, **PROTAC(H-PGDS)-1**, and **PROTAC(H-PGDS)-2**. (b) KU812 cells were incubated with the indicated concentration of the compounds for 24 h. The H-PGDS/β-Actin ratio was normalized by the vehicle control as 100. Data in the bar graph are the mean ± SD ($n = 3$). * $P < 0.01$ compared to vehicle-treated control in a two-tailed Student's t test. (c) KU812 cells were incubated with the indicated concentration of the compounds for 24 h and then incubated with 5 μM A23187 in the presence of each compound for 30 min. PGD₂ levels in the medium were measured. Data in the bar graph are the mean ± SD ($n = 3$). * $P < 0.05$ in a two-tailed Student's t test.

In conclusion, a potent degrader of the H-PGDS protein, **PROTAC(H-PGDS)-1**, was successfully developed by conjugating TFC-007 (H-PGDS ligand) to pomalidomide (E3 ligase, CRBN ligand). **PROTAC(H-PGDS)-1** effectively induced the selective degradation of H-PGDS protein via the UPS and significantly suppressed PGD₂ production. The degrader **PROTAC(H-PGDS)-1** with a new mechanism of action is expected to be as effective, or more effective, than conventional inhibitors and should allow a reduction in the number of doses for the treatment of chronic inflammation. To clinically develop an H-PGDS degrader, we need to determine the on- and off-target effects, and to investigate the ADMET (absorption, distribution, metabolism, excretion, and toxicity) aspects of the H-PGDS degrader. Further structure-based improvement of the H-PGDS degraders is in progress in our laboratory.

Methods

Synthesis and characterization of H-PGDS degraders

We designed hybrid molecules, in which an H-PGDS inhibitor was linked to a ligand for CRBN or IAPs via a linker containing a polyethylene glycol unit. The chemical synthesis and physicochemical data of the compounds are provided in the supporting information.

Reagents

Tissue culture plastics were purchased from Greiner Bio-One (Frickenhausen, Germany). Cycloheximide was purchased from Sigma-Aldrich (St. Louis, MO, USA). MG132 was purchased from Peptide Institute (Osaka, Japan). MLN7243 was purchased from Active Biochem (Maplewood, NJ, USA). Pomalidomide was purchased from

Cayman Chemical (Ann Arbor, MI, USA). A23187 was purchased from Merck KGaA (Darmstadt, Germany).

Cell culture

Human chronic myelogenous leukemia KU812 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), and 100 µg/mL kanamycin (Sigma-Aldrich). Human megakaryoblastic leukemia MEG-01s cells were cultured in Dulbecco's Modified Eagle's medium (Sigma-Aldrich) supplemented with 10% FBS and 100 µg/mL kanamycin. KU812 cells were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan) Cell Bank (JCRB0104).²¹

Western blot analysis

Cells were lysed with SDS lysis buffer (0.1 M Tris-HCl at pH 8.0, 10% glycerol, and 1% SDS). Protein concentration was measured by the BCA method (Thermo Fischer Scientific), and an equal amount of protein lysate was separated by SDS-PAGE, transferred to PVDF membranes (Millipore, County Cork, Ireland), and analyzed by western blot using the appropriate antibodies. The immunoreactive proteins were visualized using Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA), and their light emission was quantified with a LAS-3000 lumino-image analyzer (Fuji, Tokyo, Japan). The following antibodies were used: anti-H-PGDS rabbit polyclonal antibody (pAb);²² anti-mPGES-1 mouse monoclonal antibody (mAb);²³ anti-CRBN rabbit pAb (#7180) (Cell Signaling Technology, Danvers, MA, USA); anti-β-Actin

mouse mAb (A2228) (Sigma-Aldrich); anti-Cyclin B1 mouse mAb (sc-245 HRP) (Santa Cruz, Dallas, TX, USA); and anti-cIAP1 goat pAb (AF8181) (R&D Systems, Minneapolis, MN, USA).

RNA isolation and quantitative PCR

Total RNA was prepared from cells using a RNeasy kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 µg of total RNA with an oligo-dT primer using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed with an ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR GreenER (Invitrogen) with gene-specific primers. Human *36B4* mRNA was used as an invariant control. The following PCR primers were used (5' to 3'): *36B4*, GGCCCGAGAAGACCTCCTT and CCAGTCTTGATCAGCTGCACA; *H-PGDS*, TGCCGTCGCTAACTGGATAA and GAGATGCCCCCGAGAAAAAC.

Binding affinity to H-PGDS

The fluorescence polarization (FP) assay was performed using a Prostaglandin D Synthase (hematopoietic-type) FP-Based Inhibitor Screening Assay Kit - Green (600007) (Cayman Chemical).

Expression and Purification of recombinant H-PGDS

Human H-PGDS were expressed and purified as described previously.⁶ In brief, *E. coli* BL21 (DE3) cells (Thermo Fisher Scientific) were transformed with the prepared plasmids. The cells were grown in LB medium (Nacalai Tesque, Kyoto, Japan) at 37 °C,

induced with 600 μ M isopropyl β -D-1-thiogalactopyranoside (Nakalai Tesque), and then cultured further for 4 h at 37 °C. The cells were collected and disrupted by sonication in PBS containing a protease inhibitor cocktail (Roche, Basel, Switzerland). After removal of cell debris by centrifugation, the supernatant was filtered and applied onto a GSH-Sepharose 4B column (GE Healthcare UK Ltd., Buckinghamshire, England). After the resin had been washed with PBS, the protein adsorbed on the GSH-Sepharose 4B was eluted with 50 mM Tris-HCl (pH 9.0) containing 10 mM GSH. Protein concentration was determined by the BCA method (Thermo Fischer Scientific).

Inhibitory activity against H-PGDS

The activity of recombinant H-PGDS was measured by the spectrophotometric method with 1 mM 1-chloro-2,4-dinitrobenzene (Sigma-Aldrich), 1 mM glutathione (Sigma-Aldrich), and 2.8 μ g/mL recombinant human H-PGDS in 100 mM Tris-HCl (pH 8.0) in the presence 0.1 mg/ml IgG (rabbit γ -globulin, Sigma-Aldrich). The reactions were monitored in a 96 well microplate, and the enzymatic product was followed at 340 nm over 3 min at 25 °C using a microplate spectrophotometer (Multiskan FC, Thermo Fisher Scientific). The IC₅₀ values were calculated using GraphPad Prism software (version 5 for Windows).

Measurement of PG levels

PGD₂ levels in the medium were measured by using a PGD₂ MOX ELISA Kit (512011) (Cayman Chemical) according to the manufacturer's instructions. The absorbance at 405 nm was measured using an EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA).

Statistical analysis

Two-tailed Student's t tests were used to determine the significance of differences between experimental groups.

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