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Design, synthesis, and biological evaluation of novel ubiquitin–activating enzyme inhibitors

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A B S T R A C T

Ubiquitin-activating enzyme (E1), which catalyzes the activation of ubiquitin in the initial step of the ubiquitination cascade, is a potential therapeutic target in multiple myeloma and breast cancer treatment. However, only a few E1 inhibitors have been reported to date. Moreover, there has been little medicinal chemistry research on the three-dimensional structure of E1. Therefore, in the present study, we attempted to identify novel E1 inhibitors using structure-based drug design. Following the rational design, synthesis, and *in vitro* biological evaluation of several such compounds, we identified a reversible E1 inhibitor (**4b**). Compound **4b** increased p53 levels in MCF-7 breast cancer cells and inhibited their growth. These findings suggest that reversible E1 inhibitors are potential anticancer agents.

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The ubiquitin–proteasome system (UPS) is important for biological processes such as protein degradation and protein function regulation. In general, poly-ubiquitinated proteins are degraded by proteasomes, and some proteins acquire new features following ubiquitination. Therefore, ubiquitination is essential for protein homeostasis. However, aberrations in protein ubiquitination are associated with several diseases, including cancer, neurodegenerative disorders, and autoimmune diseases.[1–4] Therefore, small molecule modulators that can control the UPS are potential therapeutic agents for the treatment of such diseases.

Protein ubiquitination occurs at the lysine residues, and is mediated by the sequential reactions of ubiquitin-activating enzyme (E1), conjugating enzyme (E2), and ligase (E3).[5] The first step in this process comprises the ATP-dependent activation of ubiquitin by E1 to form a ubiquitin–E1 conjugate. The conjugate is formed by a thioester linkage between the C-terminal carboxylate of ubiquitin and the catalytic cysteine of E1. E1 then transfers the ubiquitin to E2 via a thioester exchange reaction to obtain a ubiquitin–E2 conjugate. In the final step, E3, which recognizes the substrate protein, completes protein ubiquitination by transferring ubiquitin from E2 to the substrate protein. When a protein is poly-ubiquitinated by the three enzymes, it is recognized and degraded by proteasomes. Furthermore, ubiquitinated proteins are deubiquitinated by deubiquitinating enzymes (DUBs). Accordingly, the small compounds that regulate the function of E1, E2, E3,

or DUBs should be able to adjust the level of ubiquitination. Therefore, such molecules are potential therapeutic agents for the treatment of the diseases mentioned above.[6–8]

Modulators and methodologies have been developed to control ubiquitination. Examples include small molecule inhibitors that target E3 ligases–such as MDM2[9] and inhibitor of apoptosis proteins (IAPs)[10]–or DUBs.[8,11] Much research has focused on E3 ligases or DUBs.[8] Moreover, research on protein knockdown using small molecules that target E3[12–16] or on elucidating the ubiquitin cascade[17–19] has proceeded rapidly. However, there has been little drug discovery research on the ubiquitin E1 enzyme,[6] even though it has potential as a molecular target for multiple myeloma,[20] breast cancer,[21] and non-small-cell lung cancer therapy.[22] The reported ubiquitin E1 modulators (Fig. 1) are screening hits,[23] *in silico* screening hits,[24] natural products,[25–27] or peptides based on the ubiquitin sequence,[28] and medicinal chemistry targeting ubiquitin E1 enzymes remains undeveloped.[6] Furthermore, the most widely used ubiquitin E1 inhibitor is PYR-41 (**1**)[23,29] (Fig. 1), which is an irreversible E1 inhibitor, and reversible small molecule E1 inhibitors have seldom been used in cellular or animal studies. In this context, we based the design of reversible ubiquitin E1 inhibitors on structure. Herein, we describe the identification of a novel E1 inhibitor by focusing on: (i) a design based on the three-dimensional structure of E1 and the synthesis of the designed compounds; (ii) an *in vitro* assay; and (iii) a cell-based assay.

Initially, we designed E1 inhibitors by focusing on the X-ray diffraction structures of E1 complexed with ubiquitin (Protein Data Bank (PDB) code: 4II2, Fig. 2A and B).[30] The structure suggests that

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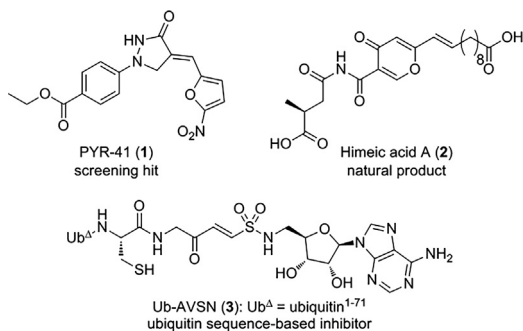 *E-mail address:* itohy@koto.kpu-m.ac.jp (Y. Itoh).

Fig. 1. Representative examples of ubiquitin-activating enzyme (E1) inhibitors.

three interactions at the active site are important for the binding of ubiquitin to E1: (i) the amide group conjugating Gly 75 with Gly 76 in ubiquitin forms a hydrogen bond with the peptide bond between Gly 560 and Thr 561 in E1; (ii) the side chain of Leu 73 in ubiquitin is positioned in a pocket formed by Leu 562, Lys 565, and Phe 888 in E1; and (iii) the side chain of Arg 72 in ubiquitin interacts with Gln 569 and Asp 584 in E1 through hydrogen

bonding. However, Leu 71 and Arg 74 do not interact with any of the E1 amino acid residues. Based on these observations, we designed compounds **4a** and **4b** (Fig. 2C–E). We expected the phenylbutanoyl amide groups in **4a** and **4b** to form hydrogen bonds with the peptide bond between Gly 560 and Thr 561. We also introduced a 4-methylpentyl group, which we expected to interact with the pocket formed by Leu 562, Lys 565, and Phe 888. We placed an amide group adjacent to the guanidino group of Arg 72 in ubiquitin to form hydrogen bonds. We designed amine compound **4c** (Fig. 2C) as a comparison to confirm the necessity of the amide group. Furthermore, we expected compound **4b**, which contains a benzoyl group, to interact with Tyr 579 through π–π interaction. To avoid the reaction with ATP, we introduced a methyl ester group to the part corresponding to the C-terminal carboxylate of ubiquitin.

Accordingly, we prepared three compounds (**4a**, **4b**, and **4c**) as shown in Scheme 1. Condensation of commercially available acid **5** with glycine methyl ester **6** gave amide **7**. A Friedel–Crafts reaction between compound **7** and 4-methyl valeryl chloride in the presence of aluminum chloride mainly produced undesired compound **8** rather than desired ketone **9**. Therefore, we reacted compound **8** with iodomethane under basic conditions to obtain
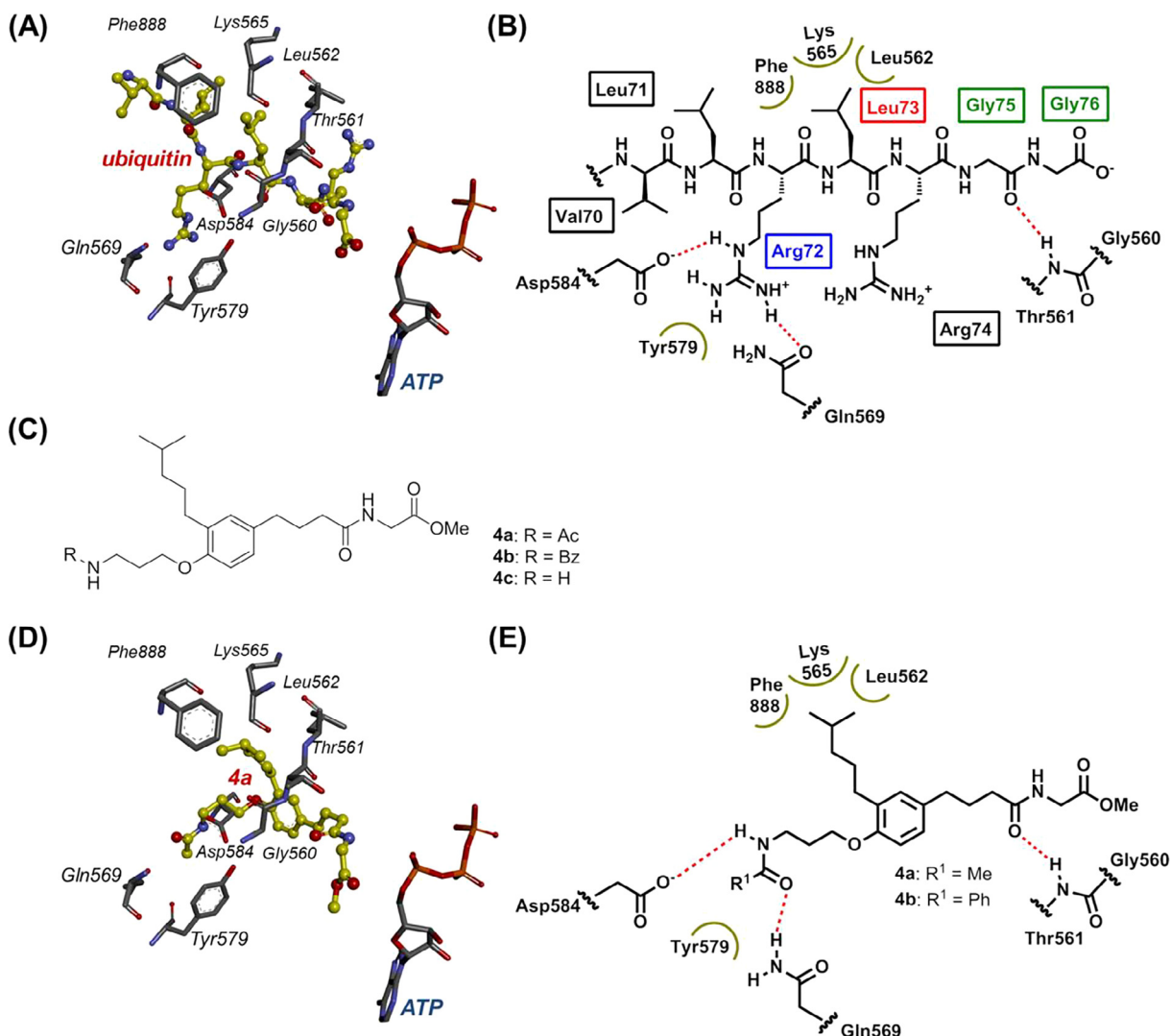

Fig. 2. (A) View of the active site of the ubiquitin-activating enzyme (E1, tube) complexed with ubiquitin (ball-and-stick; its carbon atoms: yellow) (Protein Data Bank (PDB) code: 4II2). (B) Schematic view of the binding mode of ubiquitin with E1. (C) Structures of compounds **4a**, **4b**, and **4c**. (D) The expected binding mode of compound **4a** (ball-and-stick; its carbon atoms: yellow) with E1. The compound **4a** was just placed on catalytic site of E1. (E) Schematic view of the expected binding mode of compounds **4a** and **4b** with E1.