**Regular Article**

**Screening, Synthesis, and Evaluation of Novel Isoflavone Derivatives as Inhibitors of Human Golgi β-Galactosidase**

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The genes GLBI and GALC encode GLBI isoform 1 and galactocerebrosidase, respectively, which exhibit β-galactosidase activity in human lysosomes. GLBI isoform 1 has been reported to play roles in rare lysosomal storage diseases. Further, its β-galactosidase activity is the most widely used biomarker of senescent and aging cells; hence, it is called senescence-associated β-galactosidase. Galactocerebrosidase plays roles in Krabbe disease. We previously reported a novel β-galactosidase activity in the Golgi apparatus of human cells; however, the protein responsible for this activity could not be identified. Inhibitor-derived chemical probes can serve as powerful tools to identify the responsible protein. In this study, we first constructed a cell-based high-throughput screening (HTS) system for Golgi β-galactosidase inhibitors, and then screened inhibitors from two compound libraries using the HTS system, in vitro assay, and cytotoxicity assay. An iso-flavone derivative was identified among the final Golgi β-galactosidase inhibitor compound hits. Molecular docking simulations were performed to redesign the iso-flavone derivative into a more potent inhibitor, and six designed derivatives were then synthesized. One of the derivatives, ARM07, exhibited potent inhibitory activity against β-galactosidase, with an IC\textsubscript{50} value of 14.8µM and competitive inhibition with \( K_{i} \) value of 13.3µM. Furthermore, the in vitro and cellular inhibitory activities of ARM07 exceeded those of deoxygala- lactonijirimycin. ARM07 may contribute to the development of affinity-based chemical probes to identify the protein responsible for the newly discovered Golgi β-galactosidase activity. The therapeutic relevance of ARM07 against lysosomal storage diseases and its effect on senescent cells should be evaluated further.

**Key words** β-galactosidase; inhibitor screening; isoflavone; lysosomal storage disease; senescence-associated β-galactosidase

**Introduction**

Two proteins, derived from the GLBI gene and the GALC gene, exhibit β-galactosidase activity in human cells. The GLBI gene produces lysosome-localized β-galactosidase, which is also known as GLBI isoform 1 or galactosidase beta 1. In lysosomes, GLBI isoform 1 hydrolyzes β-ketosidically linked galactopyranosyl residues from glycoproteins, sphingo-lipids, and keratan sulfate.\(^1\) Mutations in the GLBI gene result in the lysosomal storage diseases, GM1 gangliosidosis and mucopolysaccharidosis type IV.\(^2\) GM1 gangliosidosis involves progressive destruction of nerve cells in the brain and spinal cord.\(^3\) Mucopolysaccharidosis type IV involves skeletal abnormalities and cloudy corneas caused by accumulated keratan sulfate in cartilage and the cornea tissue.\(^4\) Moreover, the β-galactosidase activity of GLBI isoform 1 is the most widely used biomarker of senescent and aging cells; for example, it is used to assess replicative senescence in mammalian cells. Thus, GLBI isoform 1 is also called senescence-associated β-galactosidase (SA β-gal).\(^5,6\) Further, the GLBI gene encodes an elastin-binding protein without β-galactosidase activity, as a splicing variant, which plays functional roles in the formation of extracellular elastic fibers in the perinuclear region and cell surface.\(^7\)

In contrast, the GALC gene encodes lysosome-localized galactocerebrosidase, which hydrolyzes specific galactolipids, including galactosylceramide and psychosine.\(^8\) Certain mutations and deletions in the GALC gene significantly reduce galactocerebrosidase activity, causing damage to myelin-forming cells in the nervous system due to galactosylceramide and psychosine accumulation, ultimately resulting in Krabbe disease, a brain disorder.\(^9\)

Previously, we developed fluorogenic substrates for a β-galactosidase based on the quinone methide cleavage (QMC)-substrate design platform.\(^10–14\) and discovered unreported β-galactosidase activity in the Golgi apparatus in three human cell lines.\(^15\) This newly discovered β-galactosidase activity is entirely different from that of GLBI isoform 1 and galactocerebrosidase; however, we could not identify the protein responsible for this activity. To identify the responsible protein, we focused on the protein-maturation processes of GLBI isoform 1 and galactocerebrosidase. The maturation of GLBI isoform 1 starts from the transcription of its mRNA, which encodes 677 amino acids (AAs). The translated protein is translocated into the endoplasmic reticulum (ER) and then the signal peptide (AAs 1–23) is immediately removed. The resulting protein is then transported to the Golgi apparatus as a precursor of GLBI isoform 1 (pGLBI, AAs 24–677). Finally, the pGLBI is transported to the lysosome via the mannose-6-phosphate (M6P) pathway and is cleaved several times at the C-terminus (AAs 531–761) by lysosomal proteases to yield the mature GLBI isoform 1 (AAs 24–530), which subse-quently forms complexes with protective proteins to prevent hydrolysis by lysosomal proteases.\(^15\) In contrast, during maturation process of the nascent galactocerebrosidase protein, the...
only change occurring in the protein sequence is the removal of the signal peptide (AAs 1–42) in the ER, after which the galactocerebrosidase (AAs 43–685) is transported to lysosomes via the M6P pathway. By comparing the maturation processes of the both proteins, we realized that the GLB1 protein exists in different forms in the Golgi apparatus and lysosomes. Therefore, we hypothesized that the newly discovered Golgi \( \beta \)-galactosidase activity is exhibited by a Golgi-located pGLB1. To test this hypothesis, we aimed to perform small-molecule labeling using affinity-based chemical probes, which has been established as a powerful method for detecting target biomolecules in \textit{in vitro}, cellular, and \textit{in vivo} settings. An affinity-based chemical probe should have a high affinity and specificity for the target protein and is usually inhibitor-based.

In this study, we first constructed a cell-based high-throughput screening (HTS) system for Golgi \( \beta \)-galactosidase inhibitors using our developed fluorogenic substrate. Thereafter, target inhibitors from two compound libraries were screened, using the HTS system. Dose-response inhibition experiments were performed to further select hit compounds identified with the HTS system and in \textit{in vitro}, using recombinant pGLB1 (rpGLB1). The observation that several inhibitors were cytotoxic strongly suggests the presence of off-target proteins in the cells. Therefore, among the hit compounds, an isoflavone derivative with low cytotoxicity was selected as the final hit compound. This isoflavone derivative was redesigned into a potent inhibitor by performing molecular docking simulations against the crystallographic structure of pGLB1, and then six of the designed derivatives were synthesized to elucidate the initial structure–activity relationship (SAR) for the active site. One of them showed potent inhibitory activity \textit{in vitro}, with the activity exceeding that of the representative \( \beta \)-galactosidase inhibitor, deoxygalactonojirimycin (DGJ), and almost chemically knocked down the activity of the target enzyme in human cells. We believe that the synthesized inhibitor will contribute to the development of affinity-based chemical probes that could identify the protein responsible for the newly discovered Golgi \( \beta \)-galactosidase activity.

**Results and Discussion**

**Inhibitor Screening** The targeted Golgi \( \beta \)-galactosidase activity was previously observed in three human cell lines, HeLa cells, HT-1080 cells, and SK-N-SH cells, using QMC platform-based fluorogenic substrates. To construct the cell-based inhibitor HTS system in a 96-well format to identify inhibitors, we used HeLa cells and our developed fluorogenic substrate 1 incorporating 2-methyl TokyoGreen (2MeTG) as the fluorophore. Substrate 1 has an acetyl modification that provides cell-membrane permeability. The structure of the substrate used in this study is shown in Fig. 1. We constructed the 96-well format inhibitor HTS system based on fluorescence intensity (FI) changes due to substrate 1 hydrolysis by the Golgi \( \beta \)-galactosidase in cultured HeLa cells, according to our previous report, with modifications made to optimize the target enzyme activity. The quality and suitability of the constructed HTS system were evaluated based on the \( Z' \) factor, the coefficient of variation (CV), and the signal-to-background ratio (S/B). The \( Z' \) factor for the system was calculated to be 0.72 using the FI from substrate 1 based on evaluation of the signal and background control levels. In addition, the calculated inter-well variability CV was <8.2%, and the obtained S/B was 11.9. Together, these statistical parameters confirm the high quality and suitability of the system. The HTS system is useful for primary inhibitor screening against a compound library that especially requires high screening speed and efficiency.

The constructed cell-based HTS system was used to primarily screen two compound libraries for the Golgi \( \beta \)-galactosidase inhibitors, namely a natural compound library (NPDepo library, RIKEN) and an herbal medicine library (Institute of Natural Medicine, University of Toyama). In the primary HTS screen, we used three times the value of the standard deviation (S.D.) + average (Ave) of the inhibition rate for each inhibitor as the standard hit selection criteria and a cutoff parameter. Any compound that exhibited a more significant percentage inhibition than the cutoff parameter was declared nominally active. The first hits included 50 compounds that decreased \(<3SD + Ave\) for the negative control. In the next inhibitor screen, we eliminated compounds yielding false-positive hits from the 50 primary hit compounds. The \( Z' \) value of the inhibitor HTS was sufficiently high to exclude false-positive hit compounds; however, eliminating non-specific and/or covalent enzyme inhibitors is challenging. To remove the false-positive hit compounds from the 50 compounds, they were tested in dose–response experiments in a secondary screen using the same HTS system. Of these, 22 showed a dose–response inhibition. The details of the primary and secondary screening conditions are described in Experimental.

The HTS system did not enable further selection of secondary hit compounds as the inhibitory activity would include false positives that was mainly based on cytotoxicity. Therefore, a third screening was performed \textit{in vitro} against the hits from the second screen using commercial rpGLB1 (AAs 24–677 with a C-terminal 6-His tag) and substrate 2 (Fig. 1). In the third screen, the inhibitory activity was evaluated solely as the affinity of the inhibitor for rpGLB1. To this end, we first confirmed the \( \beta \)-galactosidase activity of rpGLB1 using substrate 2 at two pH values, pH 3.5 in lysosomes and pH 6.5 in the Golgi apparatus. The enzyme activities were evaluated by observing the FI increase due to 2MeTG release at 37°C for 60 min at 30 s intervals. The results revealed the relative hydrolysis rates, which were normalized to the amount of FI released from substrate 2 at pH 6.5 (set as 100%). Our results indicated that rpGLB1 exhibited 13.1% hydrolyzing activity at pH 3.5. Thus, rpGLB1 showed approximately eight-fold higher hydrolyzing activity at pH 6.5 than at pH 3.5. Presumably, the higher activity at pH 6.5 reflected the increased quantum yield...
of the fluorophore at an almost neutral pH, as well as differences in the conformation of rpGLB1 at either pH. Therefore, the third screening against 22 hits from the second screen was performed using rpGLB1, which had sufficient enzymatic activity at the pH of the Golgi apparatus. The inhibitory activity was evaluated by observing the decrease in FI of 2MeTG released from substrate 2 by rpGLB1 in the presence of each compound at 20 µM at a pH of 6.5. Next, the two top hits from the NPDepo library (ARM00 and 2–3F09) and the herbal medicine library (Hirsutine and Shikonin) were selected for further study. The structures of these inhibitors are summarized in Fig. 2. Furthermore, all compounds were tested in dose–response experiments at concentrations of 0.5, 12.5, and 20 µM, using the same in vitro system. All compounds except for Shikonin exhibited a dose-dependent FI inhibition. The conditions used in the third screen are described in Experimental.

Because effective inhibitors act as affinity-based chemical probes at the cellular level, it was necessary to probe whether any of the three inhibitors might interact with off-target proteins in human cells. Therefore, the cytotoxicity of the compounds was evaluated using the WST-8 method as a final screening method according to our previous report, with slight modifications. The three hit compounds, ARM00, 2–3F09, and Hirsutine, were assessed for cytotoxicity at a final concentration of 100 µM for 24 h, revealing cell survival rates of 67.8, 11.1, and 24.3%, respectively. Based on these results, we selected ARM00 as the final hit compound from among the candidate Golgi β-galactosidase inhibitors. ARM00 has been reported as one of the components of a patented sweetener, 21) and no reports are available describing its synthesis method.

**Design and Synthesis of the Inhibitor** Structurally, ARM00 has a slightly modified isoflavone scaffold. We decided to preliminarily investigate its SAR to develop more potent inhibitors. To determine the optimal chlorine substitution position of ARM00, we evaluated the rpGLB1 inhibitory activity of three commercially available positional isomers, o-isomer (ARM00), m-isomer (ARM01), and p-isomer (ARM02), at 75 µM. Moreover, the rpGLB1 inhibitory activities of three commercial compounds, ARM03–ARM05 (in which a trifluoromethyl group was introduced at the 2-position of the isoflavone skeleton of ARM00–ARM02 to enhance their interactions for pGLBI), were evaluated at 75 µM. Our results showed that the highest inhibitory activity was observed when the chlorine was substituted at the o-position. However, introducing a trifluoromethyl group into ARM00–ARM02 decreased the inhibitory activity. Briefly, none of the evaluated derivatives had greater inhibitory activity than ARM00. The structures of ARM00–ARM05 and their inhibitory activities are summarized in Fig. 3.

To enhance the inhibitory activity of ARM00, the binding mode of ARM00 to the active site of the enzyme need to be identified. However, the preliminary SAR study yielded little information. The molecular basis of the observed inhibition of pGLB1 by ARM00 was investigated by performing molecular docking simulations, which can effectively predict predominant the binding modes of a ligand located within an active site. We performed molecular docking simulations using the crystallographic structure of pGLB1 (AAs 24–677, PDB ID: 3THD,22) and ARM00 as a ligand. In the crystallographic structure, DGJ is bound to subsite −1 at the active site with a proper degree of packing (Fig. 4A). In the docking model, ARM00 was docked in the active site, with the 2-chlorobenzyl moiety being fully packed at subsite −1 (Fig. 4B).

Therefore, we designed different derivatives with substituents of appropriate sizes for subsite −1: 3-methylbutyl (ARM06), 3-methyl-2-butenyl (ARM07), and 3-methyl-2-butylnyl (ARM08). The inhibitory activities of these derivatives were expected to exceed those of ARM00. We also designed ARM09 with a methyl group as a too-small substituent and ARM10 with a 4-phenylbenzyl group as a too-large substituent, and their inhibitory activities were predicted to be lower than those of ARM00. In addition, ARM11 was designed to alleviate overcrowding at subsite −1 by removing the chlorine atom of ARM00, which could potentially increase the inhibitory activity. The initial SAR studies of these six designed derivatives were expected to reveal the binding mode of the ARM00 derivatives to the active site. However, synthesis methods for ARM00 and ARM06–ARM11 have not been reported, we synthesized...
these derivatives, according to previous reports of similar compounds. The structure and synthetic scheme of ARM00 and ARM06–ARM11 are summarized in Fig. 5. The 4-hydroxyl group of 2,4-dihydroxyacetophenone, as the starting material, was selectively protected by a tetrahydropyranyl (THP) group to obtain compound 1. Compound 1 was condensed with N,N-dimethylformamide dimethylacetal to obtain compound 2. The crude compound 2 was directly treated with I2 to furnish compound 3, which was cross-coupled with 2-methoxyphenylboronic acid by solution-phase Suzuki–Miyaura reaction to obtain compound 4. Cleavage of the THP group of 4 under acidic conditions yielded the common intermediate 5. Finally, alkylation of the phenol of 5 with corresponding alkyl halides gave ARM00 and ARM06–ARM11. All synthesized derivatives were characterized by 1H-NMR, 13C-NMR, MS analysis, and elemental analysis.

Biological Study First, we evaluated the rpGLB1 inhibitory activities of ARM00, ARM06–ARM11, and DGJ at 25 µM. The inhibition rates of ARM00 as an original compound and DGJ as a reference compound were 50.2 and 65.3%, respectively. The inhibition rates of ARM06–ARM08 in successful binding to the active site were 51.7, 71.3, and 30.4%, respectively. ARM09 and ARM10 showed the low inhibition rates of 6.1 and 20.0%, respectively, in preventing binding to the active site. ARM11 showed lower inhibitory activity than ARM00 (41.1 vs. 50.2%), despite reducing any potential steric hindrance at subsite –1. This reduction in inhibitory activity indicates that subsite –1 contains a recognition site for the substituent at the o-position. Therefore, appropriate structural modification of the o-position of the inhibitor may lead to an increase in the inhibitory activity. ARM07 was the most potent rpGLB1 inhibitor, including the original and reference compounds.

Based on these results, the ARM00 derivatives are predicted to act as reversible inhibitors of rpGLB1 through a competitive inhibition mechanism, and the 2-chlorobenzyl moiety of ARM00 and the corresponding moieties of ARM06–ARM08 and ARM11 are predicted to bind to subsite –1 at the active site. These possibilities can be tested by determining the enzyme-inhibition type and kinetic parameters of the ARM00 derivatives. Therefore, the most potent inhibitor ARM07 was selected to determine the mechanism of this inhibition type and the kinetic parameters, IC50 value and KI value. The inhibition rates of ARM07 for rpGLB1 plotted in a dose–response
In summary, we first constructed a cell-based inhibitor HTS system in a 96-well format to identify Golgi β-galactosidase inhibitors, and its quality and suitability were confirmed statistically. The HTS system, the in vitro assay, and the cytotoxicity assay were used to screen inhibitors from two compound libraries. Finally, the isoflavone derivative ARM00 was selected as a new Golgi β-galactosidase inhibitor. Initial SAR studies based on molecular docking simulations identified ARM07 as an improved inhibitor over the original hit compound ARM00 and the known inhibitor DGJ, in both in vitro and in cellular evaluations. ARM07 can be utilized to develop affinity-based chemical probes that could identify the Golgi β-galactosidase in human cultured cells, and demonstrated its potential for use as a chemical knockdown reagent for Golgi β-galactosidases at the cellular level. As a chemical knockdown reagent, it has other advantages apart from its usage in gene-knockdown studies; for instance, it is an improved alternative to using small-interfering RNAs or genome editing. In this case, ARM07 would only knock down the Golgi β-galactosidase, whereas the genetic methods would knock down both the Golgi β-galactosidase and the lysosome-located GLB1 isoform 1. This advantage should markedly help in understanding the intracellular functions of the enzyme; however, issues remain regarding the specificity of ARM07 in inhibiting β-galactosidases, pGLB1, GLB1 isoform 1, and galactocerebrosidase, and other glycosidases, which would require further detailed experimentation and discussion.

From a drug discovery viewpoint, β-galactosidases are enzymes responsible for lysosomal storage diseases, and their inhibitors can be used to enhance the activities of lysosomal enzyme-targeting drugs, which are widely used in pharmacological chaperone therapies. As a Golgi β-galactosidase inhibitor, ARM07 may also contribute to disease therapy. Additionally, pGLB1 is a precursor of both SA β-gal and GLB1 isoform 1; therefore, the relationship between senescent and aging cells and the Golgi β-galactosidase should also be explored. Our future work will focus on developing affinity-based chemical probes and identifying the protein responsible for the Golgi β-galactosidase activity to provide a basis for revealing the cellular functions of the Golgi β-galactosidase in the future.

Experimental
Chemistry New compounds were characterized by 1H-NMR, 13C-NMR, 1H-1H correlation spectroscopy, heteronuclear multiple quantum coherence spectrometry, MS, and elemental analysis. The NMR spectra were recorded with a JEOL ECA500 spectrometer (JEOL, Japan; 500 MHz for 1H and 125 MHz for 13C). Chemical shifts were expressed in ppm as downfield shifts from Me3Si. Low-resolution mass spectra were obtained with an LCMS-2020 (Shimadzu, Japan) mass spectrometer, and DUIS-2020 Dual Ion Source (DUIS) probe, which was coupled to a LC-2030C 3D (Shimadzu, Japan), was used for simultaneous electrospray ionization and atmospheric pressure chemical ionization measurements. Column chromatography was performed using Silica Gel 60N (spherical neutral particle size: 100–210µm, Kanto Chemical, Japan).

In conclusion, we first constructed a cell-based inhibitor HTS system in a 96-well format to identify Golgi β-galactosidase inhibitors, and its quality and suitability were confirmed statistically. The HTS system, the in vitro assay, and the cytotoxicity assay were used to screen inhibitors from two compound libraries. Finally, the isoflavone derivative ARM00 was selected as a new Golgi β-galactosidase inhibitor. Initial SAR studies based on molecular docking simulations identified ARM07 as an improved inhibitor over the original hit compound ARM00 and the known inhibitor DGJ, in both in vitro and in cellular evaluations. ARM07 can be utilized to develop affinity-based chemical probes that could identify the Golgi β-galactosidase in human cultured cells, and demonstrated its potential for use as a chemical knockdown reagent for Golgi β-galactosidases at the cellular level. As a chemical knockdown reagent, it has other advantages apart from its usage in gene-knockdown studies; for instance, it is an improved alternative to using small-interfering RNAs or genome editing. In this case, ARM07 would only knock down the Golgi β-galactosidase, whereas the genetic methods would knock down both the Golgi β-galactosidase and the lysosome-located GLB1 isoform 1. This advantage should markedly help in understanding the intracellular functions of the enzyme; however, issues remain regarding the specificity of ARM07 in inhibiting β-galactosidases, pGLB1, GLB1 isoform 1, and galactocerebrosidase, and other glycosidases, which would require further detailed experimentation and discussion.

From a drug discovery viewpoint, β-galactosidases are enzymes responsible for lysosomal storage diseases, and their inhibitors can be used to enhance the activities of lysosomal enzyme-targeting drugs, which are widely used in pharmacological chaperone therapies. As a Golgi β-galactosidase inhibitor, ARM07 may also contribute to disease therapy. Additionally, pGLB1 is a precursor of both SA β-gal and GLB1 isoform 1; therefore, the relationship between senescent and aging cells and the Golgi β-galactosidase should also be explored. Our future work will focus on developing affinity-based chemical probes and identifying the protein responsible for the Golgi β-galactosidase activity to provide a basis for revealing the cellular functions of the Golgi β-galactosidase in the future.
capillary apparatus and are uncorrected. ARM01-ARM05 were purchased from NAMIKI SHOJI Co., Ltd. (Japan). Compounds 1–5 were synthesized as described previously, with slight modifications. The NMR and DUIS-MS data for compounds 1, 3, 2, 4, and 5 were consistent with previously reported data.

**General Synthetic Procedure for ARM00 and ARM06–ARM11**

Common intermediate 5 (100 mg, 1 equivalent (equiv.)) was dissolved in 1 mL of acetonitrile. Corresponding alkyl halide (2 equiv.) and potassium carbonate (3 equiv.) were added, the reaction mixture was stirred at ambient temperature for 1 to 4 h, the precipitate was removed by Kiriyma filtration, and the solvent was concentrated. The obtained residue was recrystallized from hot ethanol or purified by column chromatography on silica gel (hexane: ethyl acetate = 2:1).

**7-(2-Chlorobenzoxyloxy)-3-(2-methoxyphenyl)-4H-chromen-4-one (ARM00)**

White crystalline solid (0.10 g, 68%); melting point 130–131°C; 1H-NMR (CDCl₃) δ: 3.80 (3H, s, 2'-OCH₃), 5.28 (2H, s, benzyl position), 6.95 (1H, d, J = 2.0 Hz, H-8), 6.99 (1H, d, J = 8.5 Hz, H-6), 7.02 (1H, dd, J = 1.0 Hz, J = 7.5 Hz, H-4), 7.09 (1H, dd, J = 2.0 Hz, J = 8.5 Hz, H-5), 7.10–8.8 Hz, 7.31–7.34 (4H, m, H-2', 3'-, and H-2', 3'-), 7.37 (1H, m, H-3'), 7.44 (1H, m, H-4'), 7.55 (1H, m, H-5'), 7.91 (1H, s, H-2), 8.23 (1H, d, J = 8.8 Hz, H-5), 13C-NMR (CDCl₃) δ: 55.76 (–OCH₃), 67.59 (benzyl position), 101.22 (C-8), 111.22 (C-6'), 114.78 (C-6), 118.88 (C-10), 120.56 (C-4'), 120.90 (C-1'), 122.67 (C-3), 127.13 (C-6'), 120.03 (C-5), 128.82 (C-5'), 129.45 (C-4'), 129.60 (C-3'), 131.75 (C-5'), 132.76 (C'1 or C'2'), 133.53 (C'1 or C'2'), 153.23 (C-15'), 157.42 (C-15'), 157.91 (C-9'), 162.58 (C-7'), 175.45 (–CO), DUIS-MS (positive mode): m/z = 393 [M + H]⁺ (negative mode): m/z = 391 [M – H]⁻; Anal. Calcd for C₂₁H₂₂O₄: C, 74.54; H, 6.55; N, 0.00; C, 70.32; H, 4.36; N, 0.00.

**7-(3-Methylbutyloxy)-3-(2-methoxyphenyl)-4H-chromen-4-one (ARM00)**

White crystalline solid (11 mg, 18%); 1H-NMR (CDCl₃) δ: 1.87 (2H, q, J = 2.0 Hz, –OCH₂C–), 6.97–7.01 (2H, m, H-8 and H-6), 7.02 (1H, d, J = 1.0 Hz, H-7, 7.5 Hz, H-4'), 7.03 (1H, dd, J = 2.8 Hz, J = 9.0 Hz, H-6), 7.31 (1H, dd, J = 1.5 Hz, J = 7.5 Hz, H-5'), 7.37 (1H, dd, J = 1.5 Hz, J = 7.5 Hz, H-3'), 7.92 (1H, s, H-2), 8.21 (1H, d, J = 9.0 Hz, H-5), 13C-NMR (CDCl₃) δ: 3.73 (–OCH₃), 55.76 (–OCH₃), 56.92 (–OCH₂C–), 72.95 (–C–), 84.89 (–C–), 101.51 (C-8), 111.21 (C-6'), 114.77 (C-6), 118.84 (C-10), 120.56 (C-4'), 120.92 (C-1'), 122.66 (C-3), 127.85 (C-5), 129.77 (C-3'), 131.75 (C-5'), 153.83 (C-2'), 157.55 (C-2'), 157.89 (C-8), 169.13 (C-7), 175.51 (–CO); DUIS-MS (positive mode): m/z = 321 [M + H]⁺, 343 [M + Na]⁺; Anal. Calcd for C₁₉H₁₄O₅: C, 74.99; H, 5.03; N, 0.00; O, 19.98. Found: C, 74.99; H, 5.20; N, 0.00.

**7-(3-Methylbenzoyloxy)-3-(2-methoxyphenyl)-4H-chromen-4-one (ARM09)**

White crystalline solid (91 mg, 86%); melting point 109–110°C; 1H-NMR (CDCl₃) δ: 3.80 (3H, s, 2'-OCH₃), 3.92 (3H, s, 7-OCH₃), 6.86 (1H, d, J = 2.0 Hz, H-8), 6.99 (2H, d, J = 2.0 Hz, H-8), 7.02 (1H, dd, J = 1.5 Hz, J = 7.5 Hz, H-4'), 7.31 (1H, dd, J = 1.5 Hz, J = 7.5 Hz, H-5'), 7.37 (1H, ddd, J = 1.5 Hz, J = 7.5 Hz, H-3'), 7.91 (1H, s, H-2), 8.20 (1H, dd, J = 8.5 Hz, H-5), 13C-NMR (CDCl₃) δ: 55.77 (2'-OCH₃), 55.82 (7-OCH₃), 100.16 (C-8), 111.23 (C-6'), 114.35 (C-6), 118.50 (C-10), 120.56 (C-6'), 124.97 (C-1'), 122.63 (C-3), 127.84 (C-5), 129.74 (C-3'), 131.77 (C-5'), 154.17 (C-1), 158.09 (C-2), 163.87 (C-7), 175.50 (C-5O), DUIS-MS (positive mode): m/z = 283 [M + H]⁺, 305 [M + Na]⁺; Anal. Calcd for C₁₇H₁₄O₅: C, 72.33; H, 5.00; N, 0.00; O, 22.67. Found: C, 72.32; H, 4.98; N, 0.00.

**7-(4-Phenylbenzyloxy)-3-(2-methoxyphenyl)-4H-chromen-4-one (ARM10)**

White crystalline solid (135 mg, 80%); melting point 160–161°C; 1H-NMR (CDCl₃) δ: 3.83 (3H, s, 2'-OCH₃), 5.22 (2H, s, benzyl position), 6.96 (1H, d, J = 2.5 Hz, H-8), 6.99 (1H, d, J = 8.5 Hz, H-6'), 7.02 (1H, dd, J = 1.0 Hz, J = 8.5 Hz, H-4'), 7.09 (1H, dd, J = 2.5 Hz, J = 8.5 Hz, H-6), 7.32 (1H, dd, J = 1.5 Hz, J = 9.0 Hz, H-5'), 7.35–7.39 (2H, m, H-3' and 4-phenylbenzyl), 7.44–7.47 (2H, m, 4-phenylbenzyl), 7.53 (2H, d, J = 8.0 Hz, 4-phenylbenzyl), 7.60–7.62 (2H, m, 4-phenylbenzyl), 7.64–7.66 (2H, m, 4-phenylbenzyl), 7.91 (1H, s, H-2), 8.22 (1H, d, J = 8.5 Hz, H-5), 13C-NMR (CDCl₃) δ: 55.77 (2'-OCH₃), 70.30 (benzyl position), 101.34 (C-8), 111.22 (C-6'), 114.88 (C-6), 118.71 (C-10), 120.56 (C-4'), 120.93 (C-1'), 122.65 (C-3), 127.15 (4-phenylbenzyl), 127.54 (4-phenylbenzyl), 127.96 (4-phenylbenzyl), 128.03 (4-phenylbenzyl and C-5), 128.84 (4-phenylbenzyl), 129.76 (C-3').
134.72 (4-phenylbenzyl), 140.42 (4-phenylbenzyl), 141.42 (4-phenylbenzyl), 153.81 (C-5), 157.55 (C-2), 157.51 (C-2), 153.78 (C-5), 157.51 (C-2), 157.89 (C-2), 162.86 (C-7), 175.48 (–C=O), DUlS-MS (positive mode): m/z = 435 [M + Na]⁺, 457 [M + Na]⁺, (negative mode): m/z = 433 [M–H]⁻; Anal. Cred for C₉H₆O₃: C, 80.17; H, 5.00; N, 0.00; O, 14.73. Found: C, 80.18; H, 4.91; N, 0.00.

7-Benzoxly-3-(2-methoxyphenyl)-4H-chromen-4-one (ARM11) 
White crystalline solid (0.39 g, 79%); melting point 157–158°C; 1H-NMR (CDCl₃) δ: 3.80 (3H, s, 2'-OCH₃), 5.18 (2H, s, benzyl position), 6.93 (1H, d, J = 2.0 Hz, H-8), 6.98 (1H, d, J = 7.5 Hz, H-5), 7.02 (1H, dd, J = 1.0 Hz, J = 7.5 Hz, H-4'), 7.06 (1H, dd, J = 2.0 Hz, J = 8.5 Hz, H-6), 7.32 (1H, dd, J = 2.0 Hz, J = 7.5 Hz, H-5'), 7.35–7.38 (2H, m, H-3' and H-4''), 7.41–7.47 (4H, m, H-2', H-3', H-5', and H-6'), 7.90 (1H, s, H-2), 8.21 (1H, d, J = 8.5 Hz, H-5); ¹³C-NMR (CDCl₃) δ: 55.74 (2'-OCH₃), 70.48 (benzyl position), 101.27 (C-8), 111.18 (C-6'), 114.78 (C-6), 118.64 (C-10), 120.90 (C-1'), 122.62 (C-3), 127.52 (C-2' and C-6' or C-3' and C-5'), 127.90 (C-5), 128.40 (C-4''), 128.77 (C-2' and C-6' or C-3' and C-5'), 129.74 (C-3'), 131.74 (C-5), 135.75 (C-1'), 153.78 (C-2), 157.51 (C-2'), 157.89 (C-9), 162.86 (C-7), 175.46 (–C=O); DUlS-MS (positive mode): m/z = 359 [M + Na]⁺, 381 [M + Na]⁺, (negative mode): m/z = 357 [M–H]⁻; Anal. Cred for C₉H₆O₃: C, 77.08; H, 5.06; N, 0.00; O, 17.86. Found: C, 77.06; H, 4.80; N, 0.00.

Docking Study of pGLBI versus ARMO 1000 Molecular docking simulations of pGLBI (PDB ID: 3THD, chain A) to ARMO 1000 were carried out using the Molegro Virtual Docker (version 7.0.0; Molexus, Odder, Denmark). Several preliminary steps were required before docking, such as removing water molecules, ligands, and non-targeted protein chains, and adding hydrogens. In these experiments, we used MolDock score function, which is based on a piecewise linear potential.

Primary and Secondary Compound Library Screens Both screenings were performed using the constructed HTS system against two compound libraries, a NPDepo library (RIKEN) and a herbal medicine library (Institute of Natural Medicine, University of Toyama). For the primary screening, the final concentrations of the compounds were 1 µg/well (NPDepo library) and 9.6 µM (herbal medicine library), respectively. For the secondary screening, the varying final concentrations of the primary hit compounds were 0.25, 0.5, and 1.0 µg/well (NPDepo library), and 0.096, 0.96, and 9.6 µM (herbal medicine library), respectively. The fluorescence signals from each well were recorded using Spark 10M (excitation, 480/20 nm; emission, 525/20 nm). The inhibition rate (%) of the compounds was calculated using the following equation: Inhibition rate (%) = (FIinhibitor candidate – FImax/ Avesubstrate/ AvedMSO) × 100.

In vitro β-Galactosidase Assays of rpGLBl Substrate 2 was prepared as separate 20 µM solutions in 50 mM sodium citrate buffer (pH 3.5 and 6.5, respectively). rpGLBI (recombinant human β-galactosidase-1 with a C-terminal 6His tag, 6464-GH; R&D Systems, Minneapolis, MN, U.S.A.) was prepared as separate 4 ng/µL solutions in 50 mM sodium citrate buffer (pH 3.5 and 6.5). The assays were conducted by adding rpGLBl solution (100 µL) to each substrate solution (100 µL) followed by incubation at 37°C in a 96-well plate (237105; Thermo Fisher Scientific, Waltham, MA, U.S.A.). The assays were followed by monitoring the FI changes of 2MeTG for 60 min at 30 s intervals using a multi-mode microplate reader (TECAN, Spark 10M, excitation, 480/20 nm; emission, 525/20 nm, optimized for 2MeTG). The % inhibition rates of the compounds were calculated using the above equation.

Cytotoxicity Assay HeLa cells were seeded in 96-well plates at a density of 5000 cells/well. After incubating the cells at 37°C for 24 h in 5% CO₂, the medium was replaced with 100 µL of 100 µM compound fresh medium solution con-

taining 0.5% DMSO. The cells were then incubated at 37°C for 24 h in 5% CO₂. After incubation, 10 µL of Cell Counting Kit-8 solution (CK-04, Dojindo Molecular Technology, Japan) was added to each well, and the cells were incubated at 37°C for 2 h in 5% CO₂. The absorbance at 450 nm of each well was measured using an iMark microplate reader (Bio-Rad, Hercules, CA, U.S.A.). All procedures were performed according to the manufacturer’s instructions and a previously reported method.\(^\text{20}\)

**In Vitro Inhibition Assays for SAR** A rpGLBI solution (4 ng/µL in 50 mM sodium citrate buffer, pH 6.5; 100 µL), a substrate 2 solution (20 µM in 50 mM sodium citrate buffer containing 0.67% DMSO, pH 6.5; 100 µL), and ARM00 derivatives DMSO solutions (15 mM for preliminary SAR study and 5 mM for initial SAR study; 1 µL) were added into 96-well plates, followed by incubation at 37°C in Spark 10M. The assays were followed by monitoring FI changes of 2MeTG for 120 min at 30 s intervals using Spark 10M (excitation, 480/20 nm; emission, 525/20 nm). The inhibition rate (%) of the compounds was calculated using the above equation.

**Kinetic Study of Inhibition by ARM07** The IC₅₀ value was estimated from the inhibition curve, as follows. A rpGLBI solution (4 ng/µL in 50 mM sodium citrate buffer, pH 6.5; 100 µL), a substrate 2 solution (20 µM in 50 mM sodium citrate buffer containing 0.67% DMSO, pH 6.5; 100 µL), and ARM07 DMSO solution (0.625, 1.25, or 2.5 mM) were added into 96-well plates, followed by incubation at 37°C in Spark 10M. The assays were followed by monitoring FI changes of 2MeTG for 120 min at 30 s intervals using Spark 10M (excitation, 480/20 nm; emission, 525/20 nm). The inhibition rate (%) of the compounds was calculated using the above equation.

The type of inhibition type and Kᵢ values were determined as follows. Varying concentrations of ARM07 (1.25, 2.5, and 5.0 mM in DMSO; 1 µL), varying concentrations of substrate 2 solution 10, 20, and 30 µM in 50 mM sodium citrate buffer containing 0.67% DMSO, pH 6.5, 100 µL) and rpGLBI solution (12 ng/µL in 50 mM sodium citrate buffer, pH 6.5; 100 µL) were added into 96-well plates, followed by incubation at 37°C in Spark 10M. The assays were followed by monitoring FI changes of 2MeTG for 120 min at 30 s intervals using Spark 10M (excitation, 480/20 nm; emission, 525/20 nm). The velocity of each reaction (FI/s/µg protein) was calculated by linear interpolation of the data. The inhibition type and the Kᵢ value were graphically determined with a Dixon plot.

**Cell-Based Inhibition Assay** HeLa cells were seeded in 6-well plates at a density of 9.0 × 10⁴ cells/well. After incubating the cells for 24 h, the medium was replaced with 1 mL of fresh medium. Subsequently, 5 µL of 10 mM ARM07 (final concentration 50 µM) or 10 or 60 mM DGI (final concentrations of 50 or 300 µM, respectively) in DMSO were added to each well, and the cells were incubated for 2 h. The medium was removed, and the cells were washed twice with PBS, followed by immediate fixation with 10% formalin. After washing, the cells were added with 1 mL of PBS, and then the fluorescence signals of the cells were recorded using a BIOREVO BZ-9000 fluorescence microscope (Keyence, Japan) equipped with filter sets for GFP-BP (excitation, 470/40 nm; dichroic filter, 495 nm; and emission, 535/50 nm) for Alexa Fluor 488 measurements. Fluorescence images, FIs, and FAs were analyzed using BZ-analyzer ver. 2.1 (Keyence) and WinROOF 2013 ver. 1.2.0. (Mitani, Japan) according to a previously reported method.\(^\text{12,20}\)

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

**References**