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

Kazuki Miura, Chiihiro Onodera, Motonori Takagi, Ryosuke Koyama, Takako Hirano, Toshiyuki Nishio, and Wataru Hakamata*

Department of Chemistry and Life Science, College of Bioreource Sciences, Nihon University; 1866 Kameino, Fujisawa, Kanagawa 252–0880, Japan.

Received March 2, 2020; accepted May 2, 2020

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. Galectocerebrosidase 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 isoflavone derivative was identified among the final Golgi β-galactosidase inhibitor compound hits. Molecular docking simulations were performed to redesign the isoflavone 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_{50} value of 14.8 µM and competitive inhibition with K_{i} value of 13.3 µM. Furthermore, the in vitro and cellular inhibitory activities of ARM07 exceeded those of deoxygalactonojirimycin. 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, sphingolipids, and keratan sulfate. Mutations in the GLBI gene result in the lysosomal storage diseases, GM1 gangliosidosis and mucopolysaccharidosis type IV. GM1 gangliosidosis involves progressive destruction of nerve cells in the brain and spinal cord. Mucopolysaccharidosis type IV involves skeletal abnormalities and cloudy corneas caused by accumulated keratan sulfate in cartilage and the cornea tissue. 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). 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.

In contrast, the GALC gene encodes lysosome-localized galactocerebrosidase, which hydrolyzes specific galactolipids, including galactosylceramide and psychosine. 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.

Previously, we developed fluorogenic substrates for a β-galactosidase based on the quinone methide cleavage (QMC)-substrate design platform and discovered unreported β-galactosidase activity in the Golgi apparatus in three human cell lines. 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 531–677) by lysosomal proteases to yield the mature GLBI isoform 1 (AAs 24–530), which subsequently forms complexes with protective proteins to prevent hydrolysis by lysosomal proteases. 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 β-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 in vitro, cellular, and 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 β-galactosidase inhibitors using our developed fluorogenic substrate. Therefore, 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 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 in vitro, with the activity exceeding that of the representative β-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 β-galactosidase activity.

Results and Discussion

Inhibitor Screening The targeted Golgi β-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 hydrolysis by the Golgi β-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 β-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 compounds 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 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 β-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 1 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 hit compound from among the candidate Golgi β-galactosidase inhibitors. ARM00 has been reported as one of the components of a patented sweetener, one of the components of a patented sweetener, 21) and no reports are available describing its synthesis method. However, the preliminary SAR study yielded little information. The molecular basis of the observed inhibition of 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 substrate −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 substrate −1 (Fig. 4B).

Therefore, we designed different derivatives with substituents of appropriate sizes for substrate −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 substrate −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 and biological activity. The relative inhibition rates (%) were normalized to the inhibition rate of ARM00, which was set to 100%.

**Fig. 3. Structures of the ARM00 Derivatives Tested in the Preliminary SAR Study and Their Relative Inhibition Rates**

The relative inhibition rates (%) were normalized to the inhibition rate of ARM00, which was set to 100%.

**Fig. 4. Structures of the Hit Compounds from the Third Screens**

The relative inhibition rates (%) were normalized to the inhibition rate of ARM00, which was set to 100%.

**Fig. 5. Structures of the Hit Compounds from the Third Screens**

The relative inhibition rates (%) were normalized to the inhibition rate of ARM00, which was set to 100%.
these derivatives, according to previous reports of similar compounds.\(^{23,24,25}\) 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 I\(_2\) 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 \(^1\)H-NMR, \(^{13}\)C-NMR, MS analysis, and elemental analysis.

**Biological Study** First, we evaluated the rpGLB1 inhibitory activities of ARM00, ARM06–ARM11, and DGJ at 25 \(\mu\)M. 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 \(\alpha\)-position. Therefore, appropriate structural modification of the \(\alpha\)-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, \(IC_{50}\) value and \(K_i\) value. The inhibition rates of ARM07 for rpGLB1 plotted in a dose–response...
linear curves led to an IC$_{50}$ value of 14.8 μM (Fig. 6, inset). A Dixon plot was generated to derive the $K_i$ value (Fig. 6). The plot had a common intersection at the orthant II, indicating pure competitive inhibition of rpGLB1 by ARM07, i.e., that ARM07 bound the enzyme at the active site. The $K_i$ value was calculated directly from the plot and was confirmed by plotting the 1/$V$ (FI/s/μg protein) against different ARM07 concentrations. The obtained $K_i$ value was 13.3 μM, demonstrating that ARM07 has high affinity for rpGLB1.

Next, we evaluated the Golgi β-galactosidase inhibitory activities of ARM07 and DGJ in HeLa cells in a 6-well plate. The inhibitory activities of both inhibitors were assessed by measuring changes in fluorescence signals due to enzymatic hydrolysis of substrate 1. Substrate 1 was added to the HeLa cells a final concentration of 10 μM, ARM07 was added to a final concentration of 50 μM, or DGJ was added to a final concentration of 300 μM, followed by incubation for 2 h. Next, the fluorescence signals from each well were recorded using a fluorescence microscope, and then the FI and fluorescence area (FA) of the obtained images were analyzed using an image-analysis software. The inhibition rates were calculated according to previous reports, with slight modifications. ARM07 and DGJ inhibited the FI by 74.0 and 60.9%, and the FA by 31.5 and 25.2%, respectively, indicating that ARM07 exhibited approximately more than twice inhibitory activity at a one-sixth concentration of DGJ and almost chemically knocked down the Golgi β-galactosidase activity in living HeLa cells. Moreover, ARM07 showed no cytotoxicity under these conditions. The low inhibitory activity of DGJ could be explained by its low hydrophobicity necessitating the use of a high working concentration based on its low membrane permeability. Taken together, these findings suggest that ARM07 has a high affinity for the Golgi β-galactosidase and exhibits no off-target proteins that cause cytotoxicity in HeLa cells. These considerations are of paramount importance in the development of affinity-based chemical probes that could identify the protein responsible for the Golgi β-galactosidase activity.

**Conclusion**

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 $^1$H-NMR, $^{13}$C-NMR, $^1$H–$^1$H 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 $^1$H and 125 MHz for $^{13}$C). Chemical shifts were expressed in ppm as downfield shifts from Me$_2$Si. 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: 100–210 μm, Kanto Chemical, Japan). The progress of all reactions was monitored by TLC on Silica Gel 60F$_{254}$, 0.25 mm (Merck KGA, Darmstadt, Germany). Melting points were determined with a Yamato Model MP-21.
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 1,2,3,23,24 and 5,25 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 acetone. 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 Kiriyama 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-Chlorobenzyloxy)-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-3), 6.99 (1H, d, J = 8.5 Hz, H-6), 7.02 (1H, dd, Jd = 1.0 Hz, J = 7.5 Hz, H-5'), 7.36 (1H, d, J = 1.5 Hz, J = 7.5 Hz, H-5'), 7.90 (1H, s, H-2), 8.19 (1H, d, J = 8.5 Hz, H-5); 13C-NMR (CDCl3) δ: 18.31 (–C(CH3)3), 25.86 (–C(CH3)3), 55.77 (2'-OCH3), 65.42 (–OCH2CH2–C(CH3)3), 100.92 (C-8), 111.22 (C-6'), 114.83 (C-5'), 118.38 (C-10), 118.62 (–OCH2CH=CH(CH3)2), 120.55 (C-4'), 121.00 (C-1'), 122.58 (C-3), 127.77 (C-5), 129.72 (C-3'), 131.78 (C-5'), 139.34 (C=CH2), 153.75 (C-2'), 157.55 (C-2'), 157.96 (C-9), 163.12 (C-7), 175.52 (–CO), DUIS-MS (positive mode): m/z = 377 [M + H]+, 359 [M + Na]+, (negative mode): m/z = 335 [M–H]-;

Calcd for C25H19O3: C, 72.33; H, 5.00; N, 0.00; O, 22.67.

**Chem. Pharm. Bull. Vol. 68, No. 8 (2020) 758**
134.72 (4-phenylbenzyl), 140.42 (4-phenylbenzyl), 141.42 (4-phenylbenzyl), 153.81 (C-2), 157.55 (C-2'), 157.93 (C-9), 162.88 (C-7), 175.48 (C-3 = O); DUIS-MS (positive mode): m/z = 435 [M + H]+, 457 [M + Na]+, (negative mode): m/z = 433 [M−H]−; Anal. Calcd for C34H27O7: C, 76.29; H, 4.65; N, 0.00; O, 19.06. Found: C, 76.19; H, 4.50; N, 0.00; O, 19.08.

**7-Benzoxyl-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-7), 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), 120.53 (C-4’), 121.90 (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’), 133.75 (C-1’), 135.73 (C-2’), 157.51 (C-2”), 157.89 (C-9), 162.86 (C-7), 175.46 (C-3 = O); DUIS-MS (positive mode): m/z = 359 [M + H]+, 381 [M + Na]+, (negative mode): m/z = 357 [M−H]−; Anal. Calcd for C32H25O7: C, 77.06; H, 4.80; N, 0.00.

**Docking Study of pGLB1 versus ARM10** Molecular docking simulations of pGLB1 (PDB ID: 3THD, chain A) to ARM10 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, and a re-ranking procedure was applied to the highest ranked poses to increase the docking accuracy. Affinity grid resolution was set to 0.3 Å. Ligand evaluations were based on internal energy of binding, internal H-bond formation, and Sp2–Sp2 (trigonal planar electron domain geometry) torsion angles. Candidate cavities for ligand docking were detected by including pockets with a volume of 10–10000 Å3 and a grid resolution of 0.6 Å. Among the candidate cavities, a cavity that included DGJ was selected as an active site for docking energy minimization and H-bond optimization. The number of runs was 15. Parameter settings were set to 1500 iterations, 50 max population size, 100 energy threshold, 300 max step, and 1.0 neighbor distance factor. All dockings were performed at 0.70 Å RMSD threshold.

**Cell Culture** Low-glucose Dulbecco’s modified Eagle’s medium (DMEM, D6046) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS, S1820) was purchased from MP Biomedicals (Solon, OH, U.S.A.). DMEM (D6046) was purchased from Sigma-Aldrich. The human cervical cancer cell line, HeLa (RCB0007), was purchased from MP Biomedicals (Solon, OH, U.S.A.). The Hela cells were cultured in DMEM with 10% FBS at 37°C in 5% CO2.

**Construction and Evaluation of the Cell-Based HTS System** HeLa cells were seeded in 96-well plates (Falcon, black, 353376, Corning, NY, U.S.A.) at a density of 1 × 104 cells/well. After incubating the cells for 24 h, the medium was replaced with 259 µL of fresh medium. Subsequently, 1 µL of 3mM N,N-dimethylsulfoxide (DMSO) solution of substrate 1 (11.5 µM final concentration) was added to each well, followed by incubation for 6 h. The medium was then removed, and the cells were washed three times with phosphate-buffered saline (PBS). After washing, 260 µL of PBS was added to each well, and fluorescence signals from each well were recorded on a multi-mode microplate reader (Spark 10M, excitation, 480/20 nm; emission, 525/20 nm; TECAN, Männedorf, Switzerland). Water molecules, ligands, and non-targeted protein chains, and a re-ranking procedure was applied to the highest ranked poses to increase the docking accuracy. Affinity grid resolution was set to 0.3 Å. Ligand evaluations were based on internal energy of binding, internal H-bond formation, and Sp2–Sp2 (trigonal planar electron domain geometry) torsion angles. Candidate cavities for ligand docking were detected by including pockets with a volume of 10–10000 Å3 and a grid resolution of 0.6 Å. Among the candidate cavities, a cavity that included DGJ was selected as an active site for docking energy minimization and H-bond optimization. The number of runs was 15. Parameter settings were set to 1500 iterations, 50 max population size, 100 energy threshold, 300 max step, and 1.0 neighbor distance factor. All dockings were performed at 0.70 Å RMSD threshold.

**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.}

**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/µL) 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 50 mM DGI (final concentration 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 BIORUEO EZ-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.²⁰

**Acknowledgments** We acknowledge the Chemical Resource Development Research Unit, Technology Platform Division, RIKEN Center for Sustainable Resource Science (CSRS) for providing the Natural Products Depository (NPDepo) chemical library. This research was performed as part of the Cooperative Research Project with the Institute of Natural Medicine, University of Toyama in 2016. We thank Ayako Sato for performing the elemental all analyses. This study was supported by a Sasagawa Scientific Research Grant from The Japan Science Society (Grant Number 27–304) and was supported in part by a grant from the College of Bioresource Sciences, Nihon University, Japan.

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

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

**References**

1) Suzuki Y., Sakuraba H., Oshima A., “The Metabolic and Molecular Bases of Inherited Disease,” Vol. II, ed. by S. Wulff, Elsevier Science, Amsterdam, 2000, pp. 2785–2824.
2) Hofer D., Paul K., Fantur K., Beck M., Bürger F., Caillaud C., Fumic K., Ledvina J., Lugowska A., Michelakakis H., Radeva B., Ramasswami U., Plecko B., Paschke E., Hum. Mutat., 30, 1214–1227 (2009).
3) Brunetti-Pierri N., Seaglia F., Mol. Genet. Metab., 94, 391–396 (2008).
4) Sawamoto K., Suzuki Y., Mackenzie W. G., Theroux M. W., Pizarro C., Yabe H., Orii K. E., Mason R. W., Orii T., Tomatsu S., Expert Opin. Orphan Drugs, 4, 941–951 (2016).
5) Lee B. Y., Han J. A., Im J. S., Morrone A., Johung K., Goodwin E. C., Kleijer W. J., D’Maio D., Hwang E. S., Aging Cell, 5, 187–195 (2006).
6) Xiao Y., Zhang Y., Xiao F., Drug Chem. Toxicol., 43, 213–218 (2020).
7) Privitera S., Prody C. A., Callahan J. W., Hinck A., J. Biol. Chem., 273, 6319–6326 (1998).
8) Chen Y. Q., Wenger D. A., Biochim. Biophys. Acta, 1170, 53–61 (1993).
9) Wenger D. A., Razi M. A., Luzi P., J. Neurosci. Res., 94, 982–989 (2016).
10) Hakamata W., Tamura S., Hirano T., Nishio T., ACS Med. Chem. Lett., 5, 321–325 (2014).
11) Hakamata W., Murakami K., Nishio T., Bioorg. Med. Chem., 23, 73–79 (2015).
12) Murakami K., Hakamata W., Tanaka A., Hirano T., Nishio T., Bioorg. Med. Chem., 24, 1369–1375 (2016).
13) Murakami K., Tsukagoshi T., Hirano T., Nishio T., Hakamata W., ACS Med. Chem. Lett., 26, 1309–1313 (2019).
14) Murakami K., Aoyama Y., Natsu Y., Koyama R., Hirano T., Bioorg. Med. Chem., 273, 53–61 (2018).
15) van der Spoel A., Bonten E., d’Azzo A., J. Biol. Chem., 275, 10035–10040 (2000).
16) Deane J. E., Graham S. C., Kim N. N., Stein P. E., McNair R., Cachon-González M. B., Cox T. M., Read R. J., Proc. Natl. Acad. Sci. U.S.A., 13, 15169–15173 (2011).
17) Cravatt B. F., Wright A. T., Kozarich J. W., Annu. Rev. Biochem., 77, 383–414 (2008).
18) Sletten E. M., Bertozzi C. R., Acc. Chem. Res., 44, 666–676 (2011).
19) Zhang J. H., Chung T. D., Oldenburg K. R., *J. Biomol. Screen.*, 4, 67–73 (1999).
20) Koyama R., Hakamata W., Hirano T., Nishio T., *Chem. Pharm. Bull.*, 66, 678–681 (2018).
21) Anandasankar R., Sean Michael B., U.S. Patent 20180132514 (2018).
22) Ohto U., Usui K., Ochi T., Yuki K., Satow Y., Shimizu T., *J. Biol. Chem.*, 287, 1801–1812 (2012).
23) Felpin F. X., Lory C., Sow H., Acherar S., *Tetrahedron*, 63, 3010–3016 (2007).
24) Matin A., Gavande N., Kim M. S., Yang N. X., Salam N. K., Hanrahan J. R., Roubin R. H., Hibbs D. E., *J. Med. Chem.*, 52, 6835–6850 (2009).
25) Miao J., Cui H., Jin J., Lai F., Wen H., Zhang X., Ruda G. F., Chen X., Yin D., *Chem. Commun.*, 51, 881–884 (2015).