Identification of Small-Molecule Inhibitors of Human Golgi Mannosidase via a Drug Repositioning Screen

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Three Golgi mannosidases (GMs), namely Golgi α-mannosidases IA, IB, and IC, remove mannose residues from N-glycans and regulate the quality control and transportation of nascent proteins. GM inhibitors regulate several biological events such as cell–cell communication, differentiation, and apoptosis in cancer cells. As a result, GM inhibitor-based therapies have gained significant attention for cancer treatment. However, to date, no GM inhibitor has been approved and none is in clinical development for anti-cancer treatment. Meanwhile, drug repositioning plays an important role in identifying potential inhibitors that vary in molecular structure and properties to bypass much of the early cost and time. We performed a drug repositioning screen of a compound library that included approved drugs. The estrogen receptor antagonists tamoxifen and raloxifene inhibited human GMs at the cellular level. Sulindac, a nonsteroidal anti-inflammatory drug, also inhibited GMs. Our results demonstrated the efficacy of this screening strategy and revealed lead compounds for anti-cancer drug development.

Key words  Golgi mannosidase; drug repositioning; tamoxifen; raloxifene; sulindac

The processing of N-glycans is initiated within the lumen of the endoplasmic reticulum (ER) after transfer of the glycan precursor to nascent polypeptide chains. These processes initiate the sequential cleavage of three glucose residues in the ER and four mannose residues in the cis-Golgi network. Processed high-mannose-type N-glycans undergo the addition of two N-acetylglucosamine residues and cleavage of two mannose residues in the medial-Golgi. Three Golgi mannosidases (GMs), namely Golgi α-mannosidases IA, IB, and IC, are involved in this process. GM inhibition would increase high-mannose-type N-glycan accumulation. Therefore, GM inhibitors exhibit attractive biological activities including anti-cancer, anti-virus, and immunomodulating effects as well as inhibitory effects on cell adhesion and glycoprotein disorders. GM inhibitors additionally interrupt cell–cell communication, making them promising anti-cancer drug candidates. A representative pathway of N-glycan processing and an outline of cell–cell communication are presented in Fig. 1.

Commercial GM inhibitors such as deoxymannojirimycin (DMJ), kifunensine (KIF), swainsonine (SWA), and mannostatin A have been known since the 1970s. Their structures are summarized in Fig. 2. Despite advancements in clarifying the structure–activity relationships of the known inhibitors, these inhibitors are not under development as anti-cancer drugs and no GM inhibitor has been approved for treating cancer.

Drug repositioning screening has recently emerged as an alternative approach for accelerating drug development. Following a repurposing phenotypic screen, new indications for existing drugs may be rapidly identified and clinical trials can be performed quickly, which can save both costs and time. For example, recent drug repurposing screens led to the discovery of potential new candidate therapies for cancer, sarcomas, Alzheimer's disease, cardiovascular disease, and other neurodegenerative diseases. Based on this background, we selected Golgi α-mannosidases IA, IB, and IC as the target enzymes and performed a drug repositioning screen to identify GM inhibitors from a compound library including approved drugs. To screen enzyme inhibitors, an in vitro assay system using a pure isolated enzyme for high-throughput inhibitor screening is suitable. However, there is no report on the isolation of GMs with α-mannosidase activity from human cells. Therefore, we designed, developed, and evaluated a cell-based compound-screening approach using fluorescence observation of the accumulation of high-mannose-type N-glycans as an index and utilized cell viability assays for confirmation. In this study, we present a cell-based GM-inhibitory screen system through which three approved drugs were identified as GM inhibitors at the cellular level.

Results

Development of a Cell-Based GM Inhibitor Screen System We designed a compound-screening approach to detect accumulated high-mannose-type N-glycans based on GM inhibition at the cellular level as a primary screen assay using cultured HeLa cells and a fluorescently labeled lectin, namely concanavalin A conjugated with Alexa Fluor 488 (ConA-AF488). ConA is a lectin with strong binding affinity for mannose and weak binding affinity for other sugars. Cell viability was assessed using the WST-8 method as a secondary assay to eliminate false positives based on the cytotoxicity of the candidate inhibitors. The known GM inhibitors DMJ, KIF, and SWA were used in the primary screen because they were associated with relatively high amounts of high-mannose-type N-glycans at the cellular level. Using this cell-based assay system, DMJ, KIF, and SWA were found to inhibit GMs in HeLa cells in a dose dependent manner at doses higher than 100 µM for 24 h. Changes in fluorescence intensity and area of fluorescence of the treated cells were determined using a fluorescence microscope and analysis software. These results are summarized in Fig. 5. According to
these results, cultured HeLa cells were treated with the known inhibitors at 100 µM for 24 h and accumulated high-mannose-type N-glycans were labeled with ConA-AF488. Next, the changes in fluorescence intensity and area of fluorescence of the treated cells were determined. The results illustrated that the fluorescence intensity and area of fluorescence increased by 350 and 260%, respectively, for DMJ, 258 and 199%, respectively, for KIF, and 320 and 240%, respectively, for SWA. Next, we assessed the changes in cell morphology induced by GM inhibition. In general, GM inhibition causes immature glycans, mainly high-mannose-type N-glycans, to accumulate intracellularly and induces their transport to the cell surface. As immature N-glycans have relatively weak ability to adhere to culture plates, GM inhibitor-treated cells become spherical due to surface tension. Therefore, the change in cell length in inhibitor-treated cells relative to untreated cells can indicate the extent of GM inhibition. In our experiments, 100 µM DMJ, KIF, and SWA reduced cell length by 28, 29, and 32%, respectively, for DMJ, 258 and 199%, respectively, for KIF, and 320 and 240%, respectively, for SWA. Next, we assessed the changes in cell morphology induced by GM inhibition. In general, GM inhibition causes immature glycans, mainly high-mannose-type N-glycans, to accumulate intracellularly and induces their transport to the cell surface. As immature N-glycans have relatively weak ability to adhere to culture plates, GM inhibitor-treated cells become spherical due to surface tension. Therefore, the change in cell length in inhibitor-treated cells relative to untreated cells can indicate the extent of GM inhibition. In our experiments, 100 µM DMJ, KIF, and SWA reduced cell length by 28, 29, and 32%, respectively, for DMJ, 258 and 199%, respectively, for KIF, and 320 and 240%, respectively, for SWA. Furthermore, morphological changes in inhibitor-treated cells can also be observed quantitatively. In addition, cytotoxicity was not observed in these GM inhibition assays. These data indicate that the GM-inhibitory activities are not false positives. Given these results, we considered the assay system to be suitable for cell-based GM inhibitor screening.

Cell-Based Screen of a Compound Library We conducted a GM inhibitor screen using the developed assay system and a natural compound library including pharmacologically active compounds (RIKEN, NPDepo). From the screen results, the final hits included four compounds that inhibited the three GMs and induced high-mannose-type N-glycan accumulation in HeLa cells. Next, these hits were assessed for cytotoxicity using the WST-8 method. Eventually, we identified three approved drugs, namely the estrogen receptor antagonists tamoxifen (TAM) and raloxifene (RAL) and the cyclooxygenase-1 (Cox-1) and Cox-2 inhibitor sulindac (SUL), as potential GM inhibitors. The structures of the identified inhibitors are summarized in Fig. 3. TAM, RAL, and SUL were re-evaluated using the same assay system, as summarized in Fig. 4. In cells treated with 10 µM TAM, the increases in the fluorescence intensity and area of fluorescence and the reduction in cell length were 146, 135, and 26%, respectively, versus 122, 119, and 33%, respectively, in cells treated with 10 µM RAL. In the case of SUL, no effect was observed at 10 µM treated cells. Next, in cells treated with 100 µM SUL, increases in the fluorescence intensity and area of fluorescence and the percent decline in cell length were 145, 142, and 29%, respectively. Furthermore, TAM, RAL, and SUL were not cytotoxic according to the WST-8 method. These results indicate that the screened hits inhibit GMs at the cellular level. Given these results, we have identified estrogen receptor antagonists and Cox-1/Cox-2 inhibitors as two classes of effective approved drug backbones that induce high-mannose-type
N-glycan accumulation in HeLa cells via GM inhibition. **Fluorescence Observation of High-Mannose-Type N-Glycans via Confocal Laser Microscopy** The drug repositioning screens identified TAM, RAL, and SUL as GM inhibitors. Nevertheless, the localization of high-mannose-type N-glycans following treatment with these inhibitors has not yet been investigated. To investigate the cellular localization of high-mannose-type N-glycans, we examined the distribution of these molecules using confocal laser-scanning microscopy and ConA-AF488. The results are summarized in Fig. 5. In the negative control (dimethyl sulfoxide (DMSO)), high-mannose-type N-glycans were slightly observed only in the Golgi apparatus, which included the N-glycans involved in normal N-glycan processing (Fig. 1). As expected, the known GM inhibitors DMJ and KIF increased the area of fluorescence based on an expansion of the distribution of high-mannose-type N-glycans caused by GM inhibition in the cells. In addition, high-mannose-type N-glycans were located in the Golgi apparatus as well as nearly all intracellular areas. As expected, TAM, RAL, and SUL similarly expanded the area of fluorescence as the known GM inhibitors. These results indicate that GMs represent another pharmacological target of the screened inhibitors.

**Discussion**

In this study, we first developed a cell-based GM-inhibitory assay system using fluorescence microscopy and an assessment of cytotoxicity and screened GM inhibitors from the NPDepo compound library including approved drugs using the developed assay system. Our efforts led to the identifica-
tion of the estrogen receptor antagonists TAM and RAL and the tissue Cox-1/Cox-2 inhibitor SUL as GM inhibitors. In particular, TAM and RAL inhibited GMs at a 10-fold lower concentration than DMJ and KIF, without any observed cytotoxicity. However, there is a lack of direct evidence that the increase of high-mannose-type N-glycan accumulation is through GMs inhibition. Lack of this direct proof is a major challenge for the future.

The concept of polypharmacology has only recently been recognized. According to this concept, one small-molecule drug is likely to have an average of six to seven targets.27,28 Given this fact, it is possible that TAM and RAL, which inhibited GMs and interrupted important cell–cell communication by inducing high-mannose-type N-glycan accumulation and transport, exert their anti-cancer effects by modulating biochemical networks via multiple pathways. TAM and RAL may exhibit an anticancer effect beyond an action of estrogen receptor antagonists by appropriately associating the inherent anti-breast cancer activity mechanism and GMs inhibitory activity. Our findings should significantly advance current anti-cancer research and immediately affect the development of anti-cancer therapeutics.

Experimental

Cell-based inhibitor screening against the NPDepo compound library was performed in HeLa cells using known GM inhibitors and ConA-AF488. Fluorescence signals and differential interference contrast images of the cells were recorded using a fluorescence microscope (BIOREVO BZ-9000, KEYENCE, Japan). The fluorescence intensity and area of fluorescence in the cells were analyzed using BZ-analyzer ver. 2.1 (KEYENCE) and WinROOF 2013 ver. 1.2.0 (Mitani, Japan). Cell-based imaging assays to examine the localization of high-mannose-type N-glycans were performed using known and postulated GM inhibitors and ConA-AF488 in HeLa cells. Fluorescence signals in cells were recorded using a confocal laser-scanning microscope (FluoView FV1000-D, Olympus, Japan). Cell viability assays were performed in HeLa cells using the WST-8 method. Experimental details are described in Supplementary Materials.

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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.

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