Discovery of (2-aminophenyl)methanol as a new molecular chaperone that rescues the localization of P123S mutant pendrin stably expressed in HEK293 cells

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Abstract

Pendred syndrome is the most common form of syndromic deafness. It is associated with a mutation in the SLC26A4 gene that encodes pendrin, which is thought to maintain the ion concentration of endolymph in the inner ear most likely by acting as a chloride/bicarbonate transporter. Mutations in the SLC26A4 gene are responsible for sensorineural hearing loss. In this study, we established a stable HEK293 cell line expressing P123S mutant pendrin and developed screening methods for compounds that show pharmacological chaperone activity by image analysis using CellInsight. Morphological analysis of stained cells in each well of 96-well plates yielded six compounds in the compound library. Furthermore, fluorescence intensity analysis of the intracellular localization of P123S mutant pendrin in HEK293 cells using FLUOVIEW and cytotoxicity experiments revealed that (2-aminophenyl)methanol 8 is the most promising molecular chaperone to rescue P123S mutant pendrin: the plasma membrane (M)/cytoplasm (C) ratios are 1.5 and 0.9 at the concentrations of 0.3 and 0.1 mM, respectively, and a sustained effect was observed 12 h after removal of the compound from the cell medium. Because the M/C ratio of salicylate, which was previously discovered as a molecular chaperone of P123S mutant pendrin, was approximately 1 at 10 mM concentration and a sustained effect was not observed even at 6 h, (2-aminophenyl)methanol 8 was 100 times more potent and exhibited a longer sustained effect than salicylate. These findings suggest that (2-aminophenyl)methanol 8 is an attractive candidate for therapeutic agent for Pendred syndrome patients.

1. Introduction

Pendred syndrome is the most common form of syndromic deafness and considered to account for up to 10% of all cases of syndromic hearing loss.1 Pendred syndrome is associated with a mutation in the SLC26A4 gene that encodes pendrin, which is thought to maintain the ion concentration of endolymph in the inner ear most likely by acting as a chloride/bicarbonate transporter.2–4 Pendrin is an 85.7 kDa membrane protein and a member of the anion transporter family SLC26 that maintains the ion concentration of endolymph in the inner ear, most likely by acting as a chloride/bicarbonate transporter.5–9 More than 150 mutations have been identified in the SLC26A4 gene of humans and these mutations are responsible for Pendred syndrome and non-syndromic hearing loss with an enlarged vestibular aqueduct (NSEA).10,3,11

restored in the presence of 10 mM of salicylate, revealing that salicylate acts as a molecular chaperone\textsuperscript{14,15} to rescue these pendrin mutants.\textsuperscript{13} A molecular chaperone corrects these misfolding pendrin mutants in folding and restores their function. It is therefore necessary to find compounds that exhibit the pharmacological chaperone activity for the treatment of patients with syndromic hearing loss.\textsuperscript{16,17} In this study, we established a screening method for compounds that show P123S mutant pendrin chaperone activity using P123S mutant pendrin stably expressed in human embryonic kidney (HEK) 293 cells, in hopes of finding more pharmacologically active compounds to rescue mutant pendrin.

2. Materials and methods

2.1. Expression vectors

Expression vectors of human pendrin wild-type (WT; Accession No. NM_000441.1) and P123S mutant were constructed in a previous study.\textsuperscript{13} In brief, cDNA of wild type or P123S mutant fused with 3 × FLAG tag at its C-terminus was cloned into the pcDNA3.1 mammalian expression plasmid vector. The expression plasmids were amplified in E. coli (JM109) and purified from the cells using a Plasmid Mini Kit (QIAGEN, Germantown, MD).

2.2. Cell culture and construction of stably expressed cell lines of WT and P123S pendrins in HEK293 cells

HEK293 cells were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO\textsubscript{2}. After 12 h, the cells were transfected with WT and P123S plasmid DNAs by using FuGENE\textsuperscript{\textregistered} HD transfection reagent (Promega, Madison, WI) according to the manufacturer’s protocol. These two types of cells were seeded in 10% FBS-RPMI-1640 medium containing 500 µg/ml G-418 Sulfate (Calbiochem\textsuperscript{\textregistered}, La Jolla, CA) for 10 days and neomycin-resistant cells were obtained. The neomycin-resistant cells were cloned with the limiting dilution-culture method in medium containing 500 µg/ml G-418 Sulfate (Calbiochem\textsuperscript{\textregistered}) on duplicate 96-well culture plates. The cells on one of two plates were subjected to immunofluorescence staining in the following way: After the cells were washed with PBS three times, they were fixed in 4% paraformaldehyde for 5 min. Then, the cells were blocked with 1% BSA-PBS for 1 h. Mouse anti-FLAG\textsuperscript{\textregistered} primary antibody (Sigma) was added to the cells and the cells were incubated at room temperature for 1 h and washed with PBS three times. The cells were incubated in the presence of anti-mouse secondary antibody conjugated with Alexa Fluor\textsuperscript{\textregistered} 546 (Invitrogen, Carlsbad, CA) and DAPI (Wako Pure Chemicals Ltd, Osaka, Japan) at room temperature for 1 h and then washed three times with PBS. Fluorescence images of the cells were obtained with the confocal laser scanning microscope and analyzed by FLUOVIEW\textsuperscript{\textsuperscript{\textregistered}} (Olympus, Co.).

2.3. Immunofluorescence microscopy

WT cells and P123S cells were seeded in a 96-well culture plate or a 3.5 cm culture dish containing 10% FBS-RPMI-1640 medium. P123S cells were incubated in the presence of vehicle (0.1% DMSO), salicylate, and salicylate derivatives (Fig. 1) at the specified concentration of each compound for 12 h. WT cells were incubated in the presence of vehicle (0.1% DMSO) for 12 h as positive control. Salicylate and salicylate derivatives were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MU).

The cells were subjected to immunofluorescence staining in the following way: After the cells were washed with PBS three times, they were fixed in 4% paraformaldehyde for 5 min. Then, the cells were blocked with 1% BSA-PBS for 1 h. Mouse anti-FLAG\textsuperscript{\textregistered} primary antibody (Sigma) was added to the cells and the cells were incubated at room temperature for 1 h and washed with PBS three times. The cells were incubated in the presence of anti-mouse secondary antibody conjugated with Alexa Fluor\textsuperscript{\textregistered} 546 (Invitrogen, Carlsbad, CA) and DAPI (Wako Pure Chemicals Ltd, Osaka, Japan) at room temperature for 1 h and then washed three times with PBS. Fluorescence images of the cells were obtained with the confocal laser scanning microscope and analyzed by FLUOVIEW\textsuperscript{\textsuperscript{\textregistered}} (Olympus, Co.).

2.4. Localization of P123S mutant pendrin in HEK293 cells

Immunofluorescent cells on the 96-well culture plate were analyzed by CellInsight\textsuperscript{\textsuperscript{\textregistered}} (Thermo Fisher Scientific Inc., Waltham, MA). The cells in each well were divided into 100 fields to detect approximately 100 cells in one field. Cell images were analyzed by using protocols Ch 1–4 as shown in Fig. 2. Protocol Ch 1 was programmed to recognize the plasma membrane (the outline of cells), whereas protocol Ch 2 was programmed to detect the nucleus stained by DAPI. Protocol Ch 3 was programmed to measure the

Fig. 1. Chemical structures of salicylate and its derivatives. All compounds are commercially available.
2.5. Cytotoxicity of compounds toward P123S mutant HEK293 cells

P123S mutant HEK293 cells were seeded in a 96-well culture plate at $5 \times 10^4$ cells/well and incubated at 37 °C for 12 h. Then, the cells were further incubated in RPMI-1640 medium containing compounds at various concentrations for 72 h. Cell viability was
determined by the MTT assay. The concentration required to reduce cell viability by 50% (IC\textsubscript{50}) was determined from the semilogarithmic dose-response plots. Triplicate experiments were carried out.

2.6. Sustainability evaluation of chaperone activity of compounds toward P123S mutant pendrin in HEK293 cells

The cells were seeded in a 96-well culture plate (5 × 10\textsuperscript{4} cells/well) and incubated in the presence of vehicle (0.1% DMSO), salicylate, and salicylate derivatives for 12 h. The cells were washed with PBS and then further incubated for 0, 6, and 12 h. Sustainability evaluation was carried out by measuring the time-dependent distribution of P123S mutant pendrin from the plasma membrane in HEK293 cells. Each sample was stained with anti-FLAG\textsuperscript{\textregistered} primary antibody and DAPI- and immunofluorescence-stained cells were analyzed in the same manner as that described in Section 2.3. Triplicate experiments were carried out.

3. Results and discussion

3.1. Localization analysis of P123S mutant pendrin in HEK293 cells

The localization of P123S mutant pendrin in HEK293 cells was analyzed by CellInsight\textsuperscript{\textregistered}. We first tried to determine the localization of P123S mutant pendrin in the plasma membrane directly using protocol Ch 4, which was programmed to detect translocated pendrins. As shown in Fig. 2(C), the dose-dependent increase of the fluorescence intensities of P123S mutant pendrin induced by salicylate was not observed as expected, although P123S mutant pendrin was localized in the plasma membrane of HEK293 cells induced by salicylate (see Fig. 4). Therefore, we analyzed the...
fluorescence intensities of P123S mutant pendrin in the cytoplasm of HEK293 cells using protocol Ch 3. As shown in Fig. 2(D), a dose-dependent decrease of the fluorescence intensities of P123S mutant pendrin was observed in the cytoplasm, indicating that protocol Ch 3 can be used to detect the pharmacological chaperone activity of compounds using stably expressed P123S mutant pendrin in HEK293 cells.

3.2. Effects of salicylate derivatives on localization of P123S mutant pendrin in HEK293 cells

Because we were able to establish a protocol for the determination of P123S mutant pendrin localization in the plasma membrane of HEK293 cells as discussed in the previous section, we next examined a procedure for screening salicylate derivatives with regard to their effects on the localization of P123S mutant pendrin. The results are summarized in Fig. 3. Salicylate, but not salicyl alcohol, induced the localization of P123S mutant pendrin in plasma membrane in a concentration-dependent manner. Compounds 1, 4, and 7, which have a carboxylic acid moiety in the molecules, did not show the chaperone activity, indicating that a phenolic hydroxyl group is essential for the chaperone activity. Compounds 2, 3, 6, 12, and 13 did not show the chaperone activity as well. Compounds 5 and 11, which have an electron-withdrawing group in the molecules, showed significant chaperone activity, indicating that the acidity of benzoic acids is also important for the chaperone activity. A benzene ring is also indispensable for the chaperone activity (compounds 9 and 10). Interestingly, aniline derivative 8 and boronic acid derivative 14 also showed significant chaperone activity, suggesting that carboxylic acid can be replaced by amine or boronic acid. 2-(2-Hydroxyethyl)phenol 18, but not 2-(2-hydroxyethyl)aniline 19, possessed significant chaperone activity. Compound 20, which has an ethylene linker between benzene ring and carboxylic acid in salicylate, also exhibited the chaperone activity. Among the compounds screened, compounds 5, 8, 11, 14, 15, and 18 showed significant chaperone activity to rescue P123S mutant pendrin.

3.3. Effects of salicylate derivatives on cell viability of P123S mutant HEK293 cells

Proliferation and cell viability are also an important consideration when examining the chaperone activity of compounds for the rescue of P123S mutant pendrin. Therefore, we investigated the
cytotoxicity of compounds toward P123S mutant HEK293 cells. As shown in Table 1, salicylate exhibited 4.2% inhibition of cell viability at 15 mM. The other compounds are non-cytotoxic or have low cytotoxicity similar to salicylate. Compounds 5 and 8 exhibited cytotoxicity and their IC50 values were 5.3 and 3.6 mM, respectively. Although the cell growth inhibitions of compound 8 were 43.1 and 20.8% at 1.0 and 0.3 mM, respectively, no inhibitory activity was observed at 0.1 mM concentration.

3.4. Effects of salicylate derivatives on P123S mutant pendrin translocation in HEK293 cells

We next analyzed the effects of salicylate derivatives, which were found to have more potential than salicylate in the screening, on the translocation of P123S mutant pendrin into the plasma membrane of HEK293 cells. Stable HEK293 cell line expressing pendrin mutant P123S was incubated with the salicylate derivatives in 35 mm dishes for 12 h and the cells were subjected to immunofluorescence staining for analysis under a fluorescence microscope. As compounds 11, 14, and 18 induced the aggregation of cells after incubation for 12 h, the localization of P123S mutant pendrin in HEK293 cells could not be imaged by fluorescence microscopy. These results indicated that cell aggregation could not be properly detected by CellInsight. Therefore, we analyzed the effects of compounds 5, 8, and 15 on the intracellular localization of P123S mutant pendrin in HEK293 cells. Salicylate was used as positive control. As shown in Fig. 4, P123S mutant pendrin was localized in the cytoplasm in the absence of any additives. However, it was translocated into the cell membrane in the presence of 10 mM salicylate and this localization was similar to that of wild-type pendrin in HEK293 cells. The translocation of P123S mutant pendrin into the cell membrane was also observed in the presence of 3 mM compound 5. Surprisingly, compound 8 that has no carboxylic acid in the molecule showed significant chaperone activity: the complete translocation of P123S mutant pendrin into the plasma membrane was observed even at the concentration of 0.1 mM, indicating that compound 8 possesses 100 times higher chaperone activity than salicylate. Compound 15, which has a methyl group in the molecule, showed similar chaperone activity to salicylate.

Fluorescence intensities were analyzed by FLUOVIEW (Olympus, Co.) to compare the effect of compounds on the intracellular localization of P123S mutant pendrin in HEK293 cells. The analysis was carried out on the basis of the fluorescence images in Fig. 4 and the concentration-dependent fluorescence intensities of the plasma membrane and the cytoplasm of the cells were evaluated, as shown in Fig. 5. These analyses were carried out according to our previously reported protocols. In the case of salicylate, the

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (mM)a</th>
<th>Compound</th>
<th>IC50 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>&gt;15 (4.2%)</td>
<td>10</td>
<td>&gt;15</td>
</tr>
<tr>
<td>Salicyl alcohol</td>
<td>&gt;15 (25.3%)</td>
<td>11</td>
<td>12.5 ± 2.41</td>
</tr>
<tr>
<td>1</td>
<td>&gt;15 (46.9%)</td>
<td>12</td>
<td>&gt;15 (35.5%)</td>
</tr>
<tr>
<td>2</td>
<td>&gt;15 (47.4%)</td>
<td>13</td>
<td>&gt;15 (46.8%)</td>
</tr>
<tr>
<td>3</td>
<td>10.6 ± 2.6</td>
<td>14</td>
<td>&gt;15 (22.3%)</td>
</tr>
<tr>
<td>4</td>
<td>10.2 ± 0.62</td>
<td>15</td>
<td>9.8 ± 0.48</td>
</tr>
<tr>
<td>5</td>
<td>5.3 ± 0.87</td>
<td>16</td>
<td>&gt;15 (46.7%)</td>
</tr>
<tr>
<td>6</td>
<td>&gt;15 (37.0%)</td>
<td>17</td>
<td>12.7 ± 0.85</td>
</tr>
<tr>
<td>7</td>
<td>12.7 ± 1.8</td>
<td>18</td>
<td>&gt;15 (38.3%)</td>
</tr>
<tr>
<td>8</td>
<td>3.6 ± 0.57</td>
<td>19</td>
<td>&gt;15 (35.8%)</td>
</tr>
<tr>
<td>9</td>
<td>9.7 ± 0.71</td>
<td>20</td>
<td>12.1 ± 0.96</td>
</tr>
</tbody>
</table>

a Percentage inhibition of cell viability at 15 mM is indicated in parenthesis.

Fig. 5. Fluorescence intensities of plasma membrane (M) and cytoplasm (C) of cells were evaluated by FLUOVIEW analysis in Fig. 5. M/C ratios were obtained by calculating the mean fluorescence intensities of 20 regions (0.8 mm × 0.8 mm) in plasma membrane and cytoplasm. Values are means of 6–9 cells.
fluorescence intensities of the cytoplasm (C) decreased with the increase of the fluorescence intensities of the plasma membrane (M) in a concentration-dependent manner, and the M/C ratio was approximately 1 at 10 mM concentration (Fig. 5(A), line plot). In contrast, the M/C ratio of wild-type pendrin in HEK293 cells was approximately 1.8 (data not shown). Similar to salicylate, salicylate derivatives 5, 8, and 15 also showed a concentration-dependent increase of the M/C ratios. The M/C ratio of compound 5 is approximately 1.3 at 10 mM concentration and this value is higher than that of salicylate. However, the cell growth inhibition of compound 5 is relatively high (IC₅₀: 5.3 mM) compared to salicylate. Therefore, compound 5 is not suitable as a pharmacological chaperone molecule because of its cytotoxicity. The M/C ratio of compound 15 is approximately 1.7 at 10 mM concentration, and this concentration is almost the same as its IC₅₀ (9.8 mM). In contrast, the highest M/C ratio was obtained for compound 8: it is approximately 1.8 even at 0.3 mM concentration. Furthermore, the M/C ratio of compound 8 is 0.9 at 0.1 mM concentration that is similar to that of salicylate at 10 mM concentration. Because the IC₅₀ of compound 8 is 3.6 mM, the effective concentration for the chaperone activity toward P123S mutant pendrin in HEK293 cells is still much lower than the IC₅₀.

3.5. Sustainability evaluation of chaperone activity of compounds toward P123S mutant pendrin in HEK293 cells

Finally, we investigated the sustainability of the chaperone activity of the compounds toward P123S mutant pendrin in HEK293 cells. The cells were incubated with the compounds at 10 mM (0.1 mM for compound 8) for 12 h and the compounds were removed from the cell medium. The time-dependent intracellular localization of P123S mutant pendrin in HEK293 cells was measured by fluorescence microscopy after incubation in fresh medium. The results are shown in Fig. 6. P123S mutant pendrin was localized in the cytosol of HEK293 cells, whereas it was translocated into the plasma membrane in the presence of 10 mM salicylate. However, P123S mutant pendrin was delocalized in the cytosol 6 h after salicylate was removed from the medium. Interestingly, P123S mutant pendrins treated with compounds 5, 8, and 15 were still detected in the plasma membrane 6 h after the compounds were removed from the medium. The sustainable effects were observed even after 12 h in cells treated with compounds 5 and 8. Indeed, a large population of P123S mutant pendrins were still localized near the plasma membrane in cells treated with compound 8, indicating that compound 8 is the most suitable molecular chaperone to rescue P123S mutant pendrin among the compounds screened in the current study.

4. Conclusion

We established a stable HEK293 cell line expressing pendrin mutant P123S. Using the cell line, we developed a screening method for compounds that show P123S mutant pendrin chaperone activity by image analysis using CellInsight™. Morphological analysis of stained cells in each well of 96-well plates yielded six
compounds 5, 8, 11, 14, 15, and 18 in the compound library, as shown in Fig. 1. Fluorescence intensity analysis of the intracellular localization of P123S mutant pendrin in HEK293 cells using FLUOVIEW™ and cytotoxicity experiments revealed that compound 8 possessed the most promising molecular chaperone activity to rescue P123S mutant pendrin: the M/C ratios were 1.5 and 0.9 at 0.3 and 0.1 mM concentrations, respectively. As the M/C ratio of salicylate was 1.0 at 10 mM concentration, compound 8 was able to exhibit chaperone activity toward P123S mutant pendrin at 100 times lower concentration than salicylate. Our current established high-through-put cell screening technology is based on the morphology analysis by fluorescent microscopy. Not only the integration of fluorescence but also translocation of proteins of interest can analyzed. Indeed, it has been reported that misholding mutants of membrane proteins such as V2 vasopressin receptor, P-glycoprotein and GnRH receptor can be rescued by small molecular compounds as molecular chaperones.18,16 Therefore, the current technology can be applicable in a wide variety of analysis of efficiencies of biologically active molecules including small compounds toward cell morphology including suppression and/or induction of gene expression and protein localization in cells. In addition, compound 8 showed the largest sustainable effect among the compounds screened for chaperone activity. Together, our results suggest that compound 8 is an attractive candidate for therapeutic agent for Pendred syndrome patients. Further studies of the effects of compounds on other pendrin mutations are in progress.

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References

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