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Effects of Salicylate Derivatives on Localization of p.H723R Allele Product of *SLC26A4*

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Abstract

Objective: Pendrin is a transmembrane protein encoded by the *SLC26A4* gene that functions in maintaining ion concentrations in the endolymph of the inner ear, most likely by acting as a chloride/bicarbonate transporter. Variants in the *SLC26A4* gene are responsible for sensorineural hearing loss. Although pendrin localizes to the plasma membrane, we previously identified that 8 missense allele products of *SLC26A4* were retained in the intracellular region and lost their anion exchange function. We also found that 10 mM salicylate induced the translocation of 4 out of 8 allele products from the intracellular region to the plasma membrane and restored their anion exchanger activity. However, since 10 mM salicylate exhibits cytotoxicity, the use of chemical compounds with less cell toxicity is needed. In the present study, therefore, salicylate derivatives were used as the chemical compounds and their effects on the p.H723R allele products of *SLC26A4* were investigated. Methods: HEK293 cells were transfected with the cDNA of p.H723R. Cell proliferation, viability and toxicity assays were performed to investigate the response and health of cells in culture after treatment with four types of salicylate derivatives, i.e., 2-hydroxybenzyl alcohol, 2,3-dihydroxybenzoic acid, 2’-hydroxyacetophenone and methyl salicylate. The effects of these salicylate derivatives on the localization of the p.H723R were investigated by immunofluorescence

microscopy.

Results: The application of 10 mM salicylate showed an increase in cell toxicity and decrease in cell viability, leading to a significant decrease in cell proliferation. In contrast, the application of 1 mM salicylate derivatives did not show any significant increase in cell toxicity and decrease in cell viability, corresponding to a logarithmic increase in cell concentration with an increase in culture time. Immunofluorescence experiments showed that the p.H723R retained in the endoplasmic reticulum (ER). Among the salicylate derivatives applied, 2-hydroxybenzyl alcohol induced the translocation of p.H723R from the ER to the plasma membrane 3 h after its application.

Conclusion: The results obtained showed that 2-hydroxybenzyl alcohol restored the localization of the p.H723R allele products of *SLC26A4* from the ER to the plasma membrane at a concentration of 1 mM by 3 h after its administration with less cytotoxicity than 10 mM salicylate.

Keywords: Hereditary hearing loss, Pendrin, Misfolding, Salicylate derivative

1. Introduction

Pendrin has been identified as the protein responsible for Pendred syndrome [1]. It belongs to the *solute carrier 26A* (*SLC26A*) family and consists of 780 amino acids with a molecular weight of 85.7 kDa. It is a membrane protein with 12–14 transmembrane domains [2-4] encoded by the *SLC26A4* (*PDS*) gene and is considered to function in the exchange of anions including Cl^-, HCO_3^-, I^- and $HCOO^-$ [5-10]. Pendrin is mainly expressed in the inner ear, thyroid gland and kidneys [1]. In the inner ear, pendrin has been proposed to play an important role in the exchange of Cl^- and HCO_3^- in order to maintain ion concentrations in the endolymphatic fluid [11].

More than 160 different variants in the *SLC26A4* gene have been identified in humans. These variants are responsible for Pendred syndrome, which is an autosomal recessive disorder that is characterized by sensorineural hearing loss and goiter, and also for non-syndromic hearing loss with an enlarged vestibular aqueduct (NSEVA) [1, 12-14]. Thirty-nine types of variants have been identified in the *SLC26A4* of Japanese individuals, 36% of which are c.2168A>G, leading to p.His723Arg (p.H723R) allele product, which differs from those in Caucasians [15, 16].

We previously examined the effects of salicylate on ten missense allele products of *SLC26A4* reported in Japanese patients (i.e.,p.Pro123Ser, p.Met147Val, p.Lys369Glu, p.Ala372Val, p.Asn392Tyr, p.Cys565Tyr, p.Ser657Asn, p.Ser666Phe, p.Thr721Met and p.H723R) [15, 17]. Two missense allele products, p.Lys369Glu and p.Cys565Tyr, as well as wild-type (WT) pendrin were

transported to the plasma membrane, while 8 other allele products were retained in the intracellular region. The application of 10 mM salicylate restored the cellular localization of 4 out of 8 allele products (p.Pro123Ser, p.Met147Val, p.Ser657Asn and p.H723R) from the intracellular region to the plasma membrane as well as their anion exchange activities. These findings indicated the potential of 10 mM salicylate as an effective pharmacological compound for sensorineural hearing loss caused by variants in the *SLC26A4*. However, salicylate is ototoxic [18]. We previously demonstrated that 10 mM salicylate transferred an allele product of the *SLC26A4* to the plasma membrane, whereas 1 mM salicylate did not. In the present study, we investigated some salicylate derivatives as candidates for restoring allele products of the *SLC26A* with less toxicity than salicylate.

2. Materials and methods

2.1. Expression vectors

The expression vectors of human WT pendrin (Accession No. NM_000441.1) and a p.H723R allele product (NM_000441.1:c.2168A>G) were constructed in our previous study [17]. In brief, the cDNA of WT or the p.H723R fused with the 3×FLAG tag at the C terminus was cloned into the pcDNA3.1 mammalian expression plasmid vector (Invitrogen, Carlsbad, CA). Expression plasmids were amplified in *Escherichia coli* JM109 cells (Takara Bio, Shiga, Japan) and then purified from these cells using a Plasmid Mini Kit (QIAGEN, Germantown, MD).

2.2. Cell culture and transfection

HEK293 cells (RIKEN Cell Bank, Tsukuba, Japan) were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. After 12 h, cells were transfected with 2.0 μg plasmid DNAs of WT and p.H723R and 4 μl Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After transfection for 24 h, cells were seeded on a coverslip (ϕ 18 mm, Matsunami Glass, Osaka, Japan) and placed at the bottom of each well of a 12-well plate. After an incubation for 24 h, cells were used in experiments.

2.3. Cell proliferation, viability and toxicity assays

Cell proliferation was evaluated by counting the number of cells. HEK293 cells (5×10^4 cells) were exposed to 10 mM salicylate and 1 mM 2-hydroxybenzyl alcohol (Tokyo Chemical Industry, Tokyo, Japan), 2,3-dihydroxybenzonic acid (Wako Pure Chemical Industries, Osaka, Japan), 2'-hydroxyacetophenone (Tokyo Chemical Industry) and methyl salicylate (Tokyo Chemical Industry). Cells were then incubated at 37°C with 5% CO_2 for 8 days (192 h) and their concentration was assessed every 24 h.

Cell viability was determined by measuring the dehydrogenase activity in living cells using Cell

Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. In brief, HEK293 cells were seeded on a 96-well plate at the density of 1.5×10^4 cells per well. The cells were then treated with 10 mM salicylate and four types of 1 mM salicylate derivatives mentioned above for 8 days (192 h). Every 24 h after seeding, 10 µl CCK-8 reagent was added to the wells and the cells were incubated for an additional 3 h at 37°C with 5% CO_2. The absorbance at 450 nm was measured using a microplate reader (iMark Microplate Absorbance Reader, Bio-Rad, Hercules, CA) every 24 h. The average absorbance from each set of three wells was calculated. The percent cell viability was expressed as a percentage of the control with a following equation:

$$\text{Cell viability (\%)} = \frac{A_s - A_b}{A_c - A_b} \times 100 \quad (1)$$

where A_s, A_c and A_b are the absorbance values of the sample, control (cells, culture medium and CCK-8 regent) and background (culture medium and CCK-8 reagent), respectively.

Cell toxicity was evaluated by measuring the activity of the lactate dehydrogenase (LDH) released from damaged cells in a media using Cytotoxicity LDH Assay Kit-WST (Dojindo) according to the manufacturer's instruction. As above, HEK293 cells were seeded on a 96-well plate at the density of 1.5×10^4 cells per well. The cells were then treated with 10 mM salicylate and four types of 1 mM salicylate derivatives for 8 days (192 h). Every 24 h after seeding, the culture medium was transferred to another 96-well plate and 100 µl Working Solution was added to the wells and the cells were incubated for an additional 30 min at room temperature. After the incubation, 50 µl Stop

Solution was added to the wells. The absorbance at 450 nm was measured using the microplate reader (Bio-Rad). The average absorbance from each set of three wells was calculated. The percent cell toxicity was expressed as a percentage of dead control with a following equation:

$$\text{Cell toxicity (\%)} = \frac{A_s - A_c}{A_d - A_c} \times 100 \quad (2)$$

where A_s, A_d and A_c are the absorbance values of the sample, dead control (cells, culture medium, Lysis buffer, Working Solution and Stop Solution) and control (cells, culture medium, Working Solution and Stop Solution), respectively.

2.4. Immunofluorescence microscopy

An immunofluorescence experiment was performed to analyze the localization of WT pendrin and the p.H723R in HEK293 cells. The culture medium was removed from each well of the 12-well plate and a fresh medium with 10 mM salicylate and that with 1 mM 2-hydroxybenzyl alcohol, 2,3-dihydroxybenzonic acid, 2'-hydroxyacetophenone and methyl salicylate was added. Medium was then removed after 1, 3, 6 and 12 h. Cells were washed 3 times and fixed with 4% paraformaldehyde at room temperature for 5 minutes. After washing with PBS, cells were incubated in block solution (50% Block Ace (DS Pharma Biomedical, Osaka, Japan) and 50% FBS) at 37°C for 1 h to avoid non-specific binding. After washing with PBS, cells were incubated with an anti-FLAG primary antibody (Sigma-Aldrich, St. Louis, MO) in PBS with 0.1% saponin solution at 37°C for 1 h. After washing

with PBS, cells were then incubated with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich) in PBS with 0.1% saponin solution at 37°C for 30 minutes. After washing with PBS, each coverslip was removed from the 12-well plate and placed on a glass slide. The fluorescence of cells stained with TRITC was observed using a confocal laser scanning microscope (FV500, Olympus, Tokyo, Japan).

To clarify the location at which pendrin is mainly expressed in cells, the TRITC fluorescence intensities of cells were analyzed using image analysis software (FLUOVIEW, Olympus). As shown in Fig. 1, the ratio of the fluorescence intensity of the plasma membrane I_p to that of the whole area of the cell I_w, i.e., $I_{p/w}$, was obtained by the following equation:

$$I_{p/w} = \frac{I_p}{I_w} \tag{3}$$

This calculation was performed as follows. The shape of a cell was detected on a differential interference contrast (DIC) image and the corresponding TRITC fluorescence image was trimmed along this shape. The sum of the fluorescence intensity values of all pixels within the cell was defined as I_w. The width of the plasma membrane was assumed to be 0.8 μm based on our previous study [17]. The sum of the fluorescence intensity values of all pixels within the plasma membrane area was defined as I_p.

The intracellular localization of p.H723R in HEK293 cells was also analyzed. The culture medium was removed from each well of the 12-well plate. Cells were washed 3 times and fixed with

4% paraformaldehyde at room temperature for 5 minutes. After washing with PBS, cells were incubated in block solution (1w/v% Block Ace (DS Pharma Biomedical) in PBS) at 37°C for 1 h to avoid non-specific binding. After washing with PBS, cells were incubated with anti-DYKDDDDK primary antibody (Invitrogen, Waltham, MA), ER-selective dye (ENZ-51025-K500, Enzo, Farmingdale, NY) and Hoechst 33342 nuclear staining dye (Enzo) in PBS with 0.1% saponin (Nacalai Tesque, Kyoto, Japan) solution at 37°C for 1h. After washing with PBS, cells were then incubated with a Cy3-conjugated anti-mouse IgG secondary antibody (Invitrogen) in PBS with 0.1% saponin solution at 37°C for 30 minutes. After the incubation with secondary antibody, each coverslip was washed with PBS and removed from the 12-well plate and placed on a glass slide. The fluorescence of cells stained was observed using a fluorescence microscope (BZ-X800, Keyence, Osaka, Japan).

2.5 Statistics

Cell proliferation assay were analyzed using two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* multiple comparison. For the other experiments, statistical analyses were performed using one-way ANOVA followed by Dunnett's *post hoc* multiple comparison. Data are presented in the text and Figures as the means ± SD. The *p*-value of less than 0.05 was considered significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [19], which is a graphical user interface for R (The R Foundation

for Statistical Computing, Vienna, Austria).

3. Results

3.1. Response of HEK293 cells to salicylate and its derivatives

Effects of salicylate derivatives (2-hydroxybenzyl alcohol, 2,3-dihydroxybenzonic acid, 2'-hydroxyacetophenone and methyl salicylate) on HEK293 cells was investigated by cell proliferation, cell viability and cell toxicity assays.

Figure 2 shows the cell proliferation of HEK293 cells treated with salicylate and its derivatives. When cells were treated with 10 mM salicylate, cell concentration was significantly reduced compared to the control (# $p < 0.01$ *re* Control, two-way repeated-measures ANOVA; ** $p < 0.01$ *re* 0 h, Bonferroni's *post hoc* multiple comparison after two-way repeated-measures ANOVA). In contrast, when cells were treated with 1 mM salicylate derivatives, cell concentration showed logarithmic increase with an increase in culture time. No significant difference in cell concentration was found between those cells and the control (N.S. $p = 0.1223$ *re* Control, two-way repeated-measures ANOVA).

Figure 3 shows the cell viability of HEK293 cells treated with salicylate and its derivatives. CCK-8 assay showed that 1 mM salicylate derivatives had no significant effect on HEK293 cell viability. On the other hand, 10 mM salicylate induced significant reduction in cell viability 24-192 h after treatment (*** $p < 0.001$ *re* Control, one-way ANOVA with Dunnett's *post hoc* analysis).

To further examine the cytotoxicity of salicylate and its derivatives on HEK293 cells, LDH release from damaged cells, a biochemical indicator of cytotoxicity, was quantified as shown in Fig. 4. LDH assay showed that treatment with 1 mM salicylate derivatives did not show any significant cell toxicity, while treatment with 10 mM salicylate showed significant increase in cell toxicity 24-192 h after the treatment (*** $p < 0.001$ *re* Control, one-way ANOVA with Dunnett's *post hoc* analysis).

3.2. Localization of WT pendrin and the p.H723R

An immunofluorescence experiment was performed to confirm differences in the intracellular localization of WT pendrin and the p.H723R in HEK293 cells. Figure 5 shows immunostaining micrographs of WT pendrin and the p.H723R labeled with TRITC in HEK293 cells. The left, center and right images show TRITC fluorescence, DIC and merged images, respectively. Wild-type pendrin mainly localized to the plasma membrane of cells, whereas p.H723R was not. To confirm the localization of WT pendrin and the p.H723R, cells were then co-stained with the ER marker and nuclear staining dye. In Fig. 6, upper, middle and lower panels show the cells expressing WT pendrin, those expressing p.H723R and the cells without expression vector (negative control). As shown in this figure, WT pendrin was localized to the plasma membrane while p.H723R was mostly localized to the ER. The expression of pendrin was not confirmed from the cells in the negative control (w/o expression vector).

3.3. Effects of salicylate and its derivatives on the localization of the p.H723R

We previously demonstrated that the p.H723R was transferred from the intracellular region to the plasma membrane of cells 12 h after the administration of 10 mM salicylate [17]. To clarify the stage at which the salicylate-induced movement of the p.H723R occurred after its administration, the localization of the p.H723R was investigated 1, 3, 6 and 12 h after the application of 10 mM salicylate and 1 mM salicylate derivatives. As shown in Fig. 7, the p.H723R mainly localized to the intracellular region of cells 1 h after salicylate treatment. Although a portion of the p.H723R was retained in the intracellular region 3 h after the administration of salicylate, the TRITC fluorescence intensity in such region decreased compared with that at 1 h. The p.H723R localized to the plasma membrane 6 and 12 h after the administration of salicylate, similar to WT pendrin.

To investigate the effects of four types of salicylate derivatives on the p.H723R, the localization of the p.H723R was investigated 1, 3, 6 and 12 h after their administration. As shown in Fig. 7, the p.H723R localized to the intracellular region when 1 mM 2-hydroxybenzyl alcohol was applied for 1 h. In contrast, it was mainly localized to the plasma membrane 3, 6 and 12 h after the application. As shown in Fig. 8, although the p.H723R was retained in the intracellular region until 3 h after the application of 2,3-dihydroxybenzoic acid, it was likely that the fluorescence intensity in the plasma membrane increased 6 h after the application. However, such tendency was not observed 12 h after

the application. The pH723R was retained in the intracellular region with the application of 2'-hydroxyacetophenone and methyl salicylate.

To quantify the results obtained in immunofluorescence experiments, an intensity analysis of the intracellular localization of the pendrin protein was performed. $I_{p/w}$ of HEK293 cells expressing WT pendrin and the p.H723R were calculated from immunostaining micrographs and the means and standard deviations of $I_{p/w}$ are summarized in Fig. 9.

$I_{p/w}$ significantly increased in cells expressing the p.H723R 3 h after the application of 10 mM salicylate, indicating that the p.H723R was transferred from the ER to the plasma membrane.

When cells expressing the p.H723R were treated with 1 mM 2-hydroxybenzyl alcohol, a significant increase was observed in $I_{p/w}$ 3, 6 and 12 h after its application, similar to that in cells expressing WT pendrin. Therefore, the treatment with 1 mM 2-hydroxybenzyl alcohol appeared to induce the transfer of the p.H723R from the ER to the plasma membrane 3 h after its application.

When 1 mM 2,3-dihydroxybenzoic acid was applied to cells expressing the p.H723R, $I_{p/w}$ at 6 h was statistically larger than that in cells expressing the p.H723R without the treatment, but was not at the same level as that in cells expressing WT pendrin. On the other hand, no significant difference was observed at 1, 3 and 12 h. 1 mM 2,3-dihydroxybenzoic acid appeared to induce the movement of the p.H723R; however, its transport capability was limited and/or insufficient.

The treatment with 1 mM 2'-hydroxyacetophenone and methyl salicylate did not increase $I_{p/w.}$

4. Discussion

Pendrin is a transmembrane protein that is crucial for hearing. Variants in the *SLC26A4* have been proposed to induce misfolding of the pendrin protein [14]. This may induce the accumulation of the allele product of the *SLC26A4* in the ER, cytoplasm and/or plasma membrane and prevent pendrin from exerting its original function. In other studies, variants in the *SLC26A4* have been proposed to increase susceptibility of cells to cellular stress, leading to progressive cell death [20, 21]. Recently, this susceptibility has been found to be reduced by administration of low-dose rapamycin, contributing to treatment of progressive hearing loss in Pendred syndrome patients [21]. Although these phenomena are thought to relate to mechanisms underlying hearing loss and its rescue [14, 17, 20-23], it is still controversial.

Prestin is a membrane protein of outer hair cells [24, 25]. The gene coding prestin is known as *SLC26A5*, which belongs to the same *SLC26A* gene family as pendrin. We previously demonstrated the translocation of some allele products of the *SLC26A5* from the plasma membrane to the intracellular region. We also found that they were restored from the intracellular region to the plasma membrane by the application of 10 mM salicylate [26], suggesting that salicylate may have a function to restore the localization of the allele products of the *SLC26A4*. In our previous study, we confirmed that the intracellular localization and anion exchanger activity of 4 allele products (i.e.,p.Pro123Ser,

p.Met147Val, p.Ser657Asn and p.H723R) were indeed restored by salicylate [17]. However, salicylate is known to exhibit ototoxicity [18] and we also observed that it affected the cell viability [17]. Therefore, efforts to identify other candidates with less toxicity are indispensable.

In the present study, we selected four types of chemical compounds from salicylate derivatives, i.e., 2-hydroxybenzyl alcohol, 2,3-dihydroxybenzonic acid, 2'-hydroxyacetophenone and methyl salicylate, and investigated their effects on cell proliferation, cell viability and cell toxicity. Figure 2 shows changes in cell concentration with or without these chemical compounds. Although cells with 10 mM salicylate did not grow, those treated with 1 mM salicylate derivatives showed similar growth patterns to that of the control. These data suggest that the cytotoxicity of these derivatives within this concentration may be negligibly small.

To confirm this, cell toxicity of the salicylate derivatives on HEK293 cells was quantitatively investigated using Cytotoxicity LDH Assay Kit-WST (Fig. 4) along with cell viability assay using CCK-8 (Fig. 3). When 10 mM salicylate was applied to the cells, cell toxicity increased to about 80% by 48 h after the application and remained constant until 96 h (Fig. 4). It then started to decrease to about 50%. This is an apparent decrease caused by continuous increase in absorbance value of dead control A_d and discontinuation of increase in absorbance value of sample A_s, which appear in Eq. (2). Since A_d and A_s are included in the denominator and numerator of Eq. (2), respectively, such situation leads to a decrease in the cell toxicity. The increase in A_d was caused by the increase in the

number of cells because the dead control was not treated with any chemicals until just before the measurement, while the number of cells in the sample did not increase and most of the cells were damaged due to the toxicity of salicylate, resulting in the discontinuation of increase in the absorbance. This is therefore thought to be a methodological limitation of this experiment. Cell viability started to decrease by 24 h after application of salicylate and reached to 0% at 48 h (Fig. 3). When 1 mM salicylate derivatives were applied to the cells, by contrast, significant increase in cell toxicity and decrease in cell viability were not observed compared to those in the control, as shown in Fig. 4 and Fig. 3, respectively. Considering these results into account, the cytotoxicity of the salicylate derivatives applied in the present study (i.e., 2-hydroxybenzyl alcohol, 2,3-dihydroxybenzonic acid, 2'-hydroxyacetophenone and methyl salicylate) to HEK293 cells was negligibly small within the concentration of 1 mM.

Although it has been confirmed that 1 mM salicylate derivatives examined did not show cytotoxicity, it is still unclear whether they have ototoxicity or not. Experiments using animals, cochlear explant culture systems, cochlear cell lines like HEI-OC1 [27, 28], induced pluripotent stem (iPS) cells [20, 21] and so on should be necessary to assess the ototoxicity of these chemical compounds in the future work.

The effects of salicylate derivatives on the localization of the p.H723R were then examined. In our previous study [17], we confirmed that p.H723R without salicylate or other compounds retained

in the intracellular region and this localization was not changed for 12 h (vehicle control experiment). A treatment with 10 mM salicylate for 12 h induced the transfer of the p.H723R from the intracellular region to the plasma membrane; however, the findings obtained did not show when this transfer was complete. Therefore, the localization of pendrin was investigated 1, 3, 6 and 12 h after the administration of the salicylate derivatives. As shown in Fig. 7, the p.H723R was transferred to the plasma membrane 6 h after the administration of 10 mM salicylate and 3 h after the application of 1 mM 2-hydroxybenzyl alcohol. The other three derivatives, namely, 2,3-dihydroxybenzonic acid, 2'-hydroxyacetophenone and methyl salicylate, did not induce the translocation of the p.H723R (Fig. 8).

As shown in Fig. 6, p.H723R mostly retained in the ER in this HEK expression system. When the cells were treated with salicylate and 2-hydroxybenzyl alcohol, the ratio of fluorescence intensity at the plasma membrane to that in the whole area of the cell, $I_{p/w}$, significantly increased as shown in Fig. 9. These results may imply that the localization of the p.H723R was restored from the ER to the plasma membrane 3 h after the administration of 10 mM salicylate and 1 mM 2-hydroxybenzyl alcohol. On the other hand, although $I_{p/w}$ in cells significantly increased 6 h after the application of 2,3-dihydroxybenzoic acid, it was lower than that of WT pendrin and no longer significant thereafter. This result suggests that 1 mM 2, 3-dihydroxybenzoic acid induced the translocation of the p.H723R; however, its effects appeared to be insufficient and temporary at this concentration. We previously reported that the salicylate-induced movement of the allele products of the *SLC26A4* and *SLC26A5*

was dependent on its concentration [17, 26]. Therefore, a possibility that a higher concentration of 2,3-dihydroxybenzoic acid restore the localization of the p.H723R protein to the same level as that of WT pendrin cannot be ruled out. This hypothesis will be confirmed in a future study.

In the present study, three compounds, i.e., salicylate, 2-dihydroxybenzyl alcohol and 2, 3-dihydroxybenzoic acid, restored the localization of p.H723R from the ER to the plasma membrane. As shown in Fig. 10, these compounds have 1, 3-propanediol in their structure (shaded region). This component may be of importance for restoring the p.H723R to the plasma membrane, thus, warrants further study.

In our previous study, a treatment with salicylate for 12 h restored not only the localization of four types of allele products (p.Pro123Ser, p.Met147Val, p.Ser657Asn and p.H723R), but also their anion exchanger activities. Although the effects of salicylate derivatives on the anion exchanger activities of the p.H723R were not investigated in the present study, it seems to be possible that 2-hydroxybenzyl alcohol and 2, 3-dihydroxybenzoic acid also restore anion exchanger activity of p.H723R.

DNA variants often change the amino acid composition of proteins, leading to protein misfolding. Abnormal protein folding has been implicated in the pathogenesis of various diseases [29, 30], including Pendred syndrome and non-syndromic hearing loss with NSEVA due to variants in the *SLC26A4* [22]. Previous studies indicated the restored localization of misfolded proteins and their functions following the application of chemicals such as chaperone molecules [31-34].

Pharmacological rescue by chemical compounds is one candidate for salvaging defective proteins. For example, the V2 vasopressin receptor, P-glycoprotein and GnRH were rescued using this approach [35]. Chemical compounds that refold mutated pendrin proteins correctly with low toxicity, such as 2-hydroxybenzyl alcohol in the present study, have potential as candidate drugs for the treatment of pendrin-related hereditary hearing loss.

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Disclosure statement

The authors declare no conflict of interest.

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Fig. 1. Analysis of the localization of the p.H723R allele product of *SLC26A4*. To clarify the intracellular localization of this product, two regions were defined in a cell area, i.e., the intracellular region and plasma membrane. The total TRITC fluorescence intensity of the whole area of the cell, I_w, and that of the plasma membrane, I_p, were measured. The ratio of I_p to I_w, $I_{p/w}$, was then calculated and used as an index of the localization of the allele product. In the present study, the width of the plasma membrane was assumed to be 0.8 μm [17].

Fig. 2. Cell proliferation of HEK293 cells treated with 10 mM salicylate and 1 mM its derivatives. No significant difference was observed in concentrations of cells between control cells and cells treated with salicylate derivatives (N.S. $p = 0.1223$ *re* Control, two-way repeated-measures ANOVA). In contrast, cells treated with salicylate showed significant difference in cell concentration to control (## $p < 0.01$ *re* Control, two-way repeated-measures ANOVA; ** $p < 0.01$ *re* 0 h, Bonferroni's *post hoc* multiple comparison after two-way repeated-measures ANOVA).

Fig. 3. Cell viability of HEK293 cells treated with 10 mM salicylate and 1 mM its derivatives. Salicylate derivatives had no significant effect on HEK293 cell viability, whereas 10 mM salicylate induced significant reduction in cell viability 24-192 h after treatment (*** $p < 0.001$ *re* Control, one-way ANOVA with Dunnett's *post hoc* analysis).

Fig. 4. Cell toxicity of HEK293 cells treated with 10 mM salicylate and 1 mM its derivatives. Treatment with 1 mM salicylate derivatives did not show any significant cell toxicity, while treatment with 10 mM salicylate showed significant increase in cell toxicity 24-192 h after the treatment (*** $p < 0.001$ *re* Control, one-way ANOVA with Dunnett's *post hoc* analysis).

Fig. 5. Immunostaining micrographs of WT pendrin and the p.H723R in HEK293 cells. TRITC fluorescence indicates WT pendrin and the p.H723R. The scale bar is 10 μm.

Fig. 6. Cellular localization of pendrin and p.H723R allele products of *SLC26A4* in HEK293 cells. Upper and middle panels show the cells expressing WT pendrin and p.H723R, respectively. The lower panels show the cells without expression vector (negative control). FLAG-tagged WT pendrin and p.H723R were stained with Cy3 (red). The ER and nucleus were stained with ER-marker (green) and Hoechst 33342 (blue), respectively. WT pendrin was localized to the plasma membrane while p.H723R was mostly localized to the ER. The scale bars are 20 μm and those in the magnified images are 5 μm.

Fig. 7. Immunostaining micrographs of the p.H723R in HEK293 cells incubated with salicylate and 2-hydroxybenzyl alcohol. Images were obtained at 1, 3, 6 and 12 h after their administration. The scale bar shows 10 μm.

Fig. 8. Immunostaining micrographs of the p.H723R in HEK293 cells incubated with 2,3-dihydroxybenzoic acid, 2'-hydroxyacetophenone and methyl salicylate. Images were obtained at 1, 3, 6 and 12 h after their administration. The scale bar shows 10 μm.

Fig. 9. $I_{p/w}$ of HEK293 cells expressing WT pendrin and the p.H723R treated with salicylate and its derivatives. The restored localization of the p.H723R from the cytoplasm to the plasma membrane was confirmed 3 h after the administration of 10 mM salicylate and 1 mM 2-

hydroxybenzyl alcohol (**$p < 0.01$ vs. the p.H723R without the treatment, black bar). $I_{p/w}$ of cells increased 6 h after the application of 1 mM 2,3-dihydroxybenzoic acid (*$p < 0.05$ vs. the p.H723R without the treatment, black bar); however, it was lower than that of cells expressing WT pendrin. The numbers in each bar indicate the number of samples.

Fig. 10. Structural formulae of salicylate and its derivatives. Salicylate, 2-hydroxybenzyl alcohol and 2,3-dihydroxybenzoic acid, which induced the restoration of the p.H723R, contain 1,3-propanediol in their structures (shaded region).

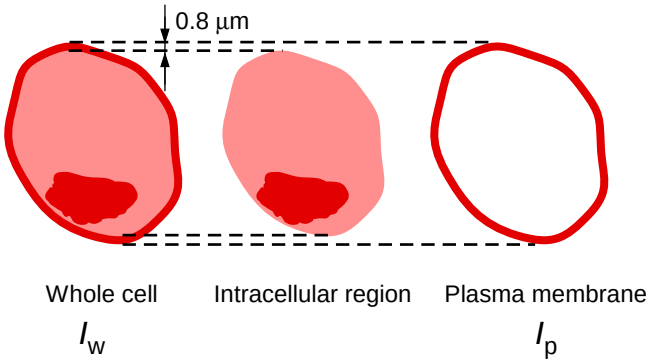

0.8 µm
Whole cell
Intracellular region
Plasma membrane
I_w
I_p

Fig. 1

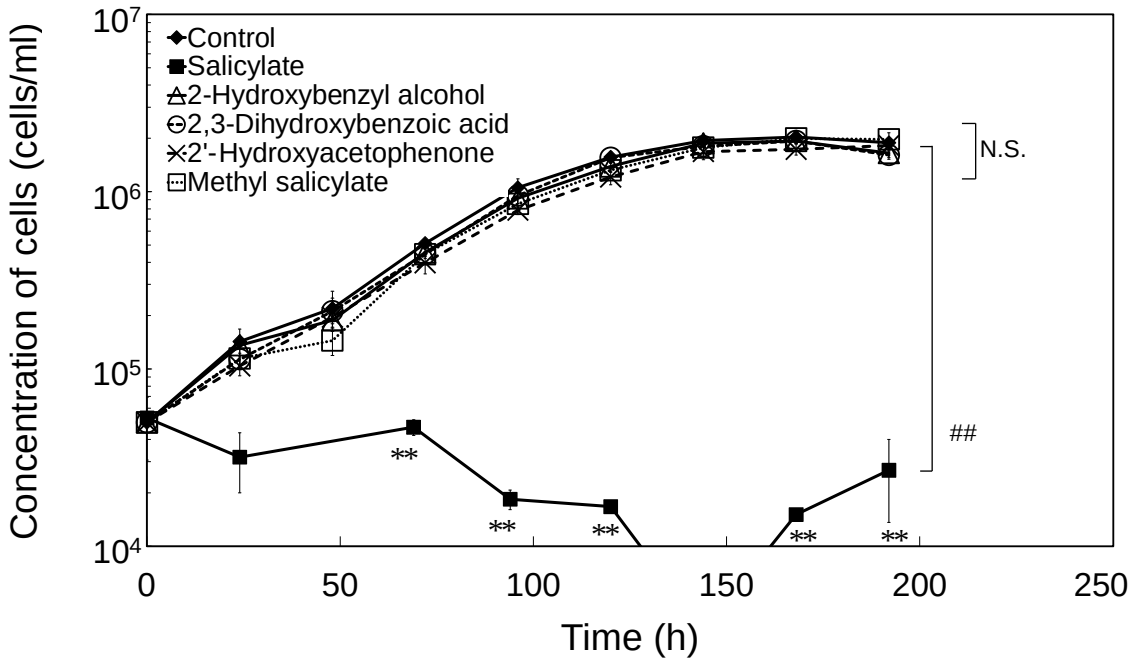

Concentration of cells (cells/ml)
10^7
10^6
10^5
10^4
Control
Salicylate
2-Hydroxybenzyl alcohol
2,3-Dihydroxybenzoic acid
2'-Hydroxyacetophenone
Methyl salicylate
N.S.
##
**
0
50
100
150
200
250
Time (h)

Fig. 2

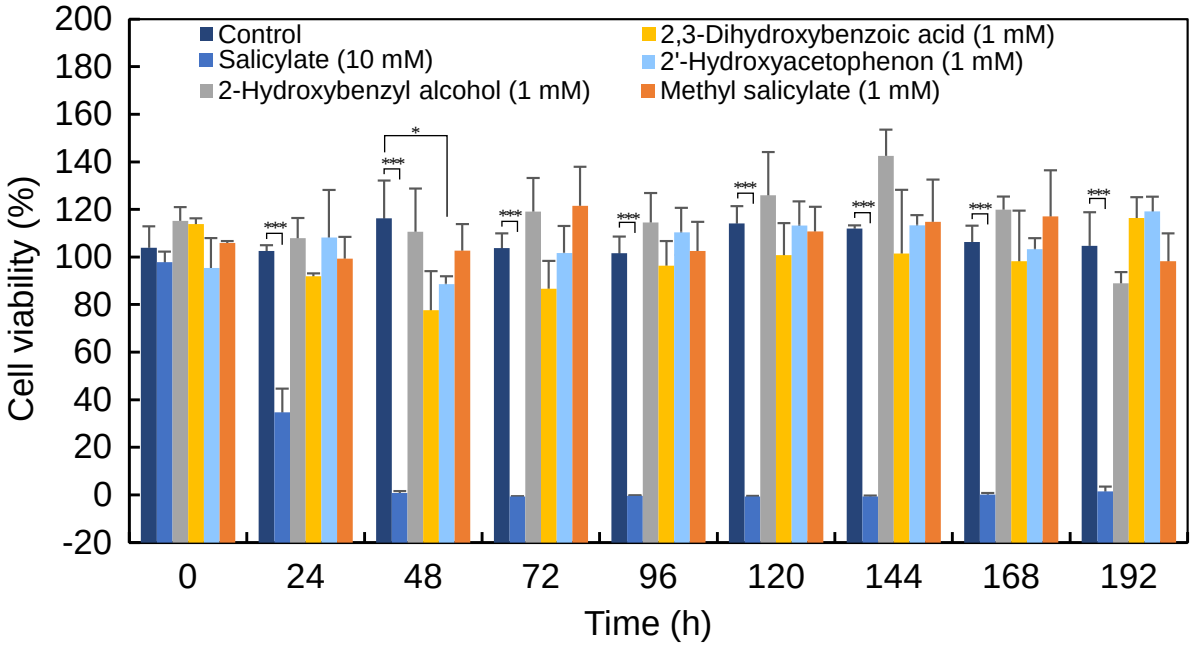

Control
Salicylate (10 mM)
2-Hydroxybenzyl alcohol (1 mM)
2,3-Dihydroxybenzoic acid (1 mM)
2'-Hydroxyacetophenon (1 mM)
Methyl salicylate (1 mM)
Cell viability (%)
200
180
160
140
120
100
80
60
40
20
0
-20
0
24
48
72
96
120
144
168
192
Time (h)
*
**
***

Fig. 3

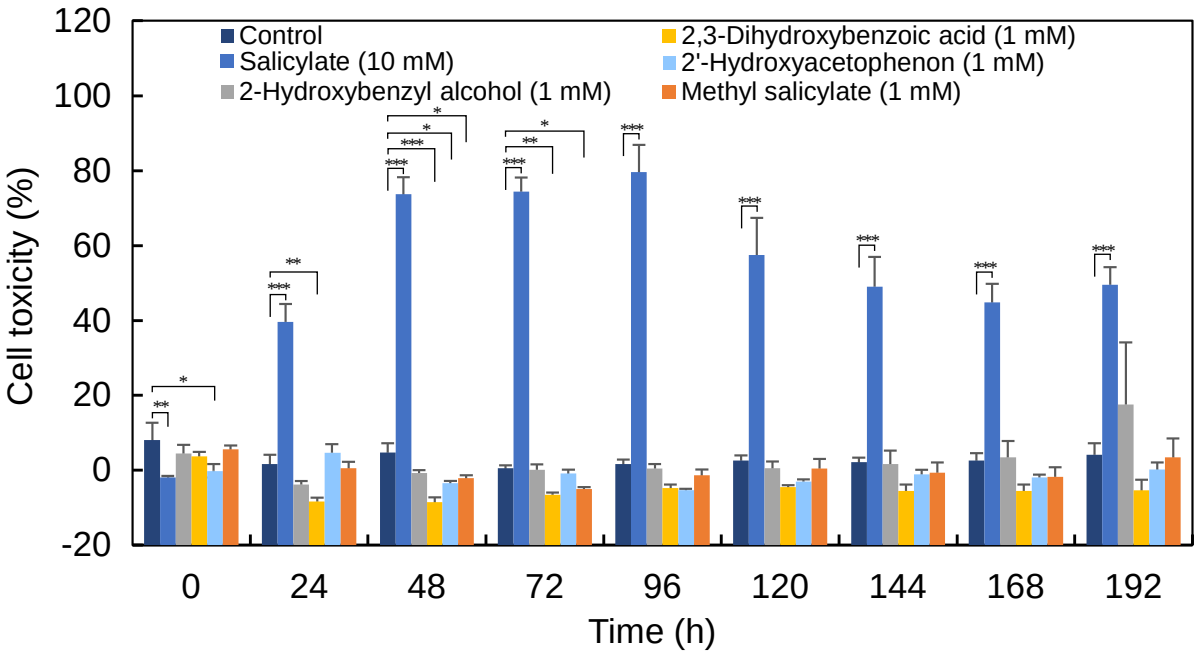

Control
Salicylate (10 mM)
2-Hydroxybenzyl alcohol (1 mM)
2,3-Dihydroxybenzoic acid (1 mM)
2'-Hydroxyacetophenon (1 mM)
Methyl salicylate (1 mM)
Cell toxicity (%)
120
100
80
60
40
20
0
-20
0
24
48
72
96
120
144
168
192
Time (h)

Fig. 4

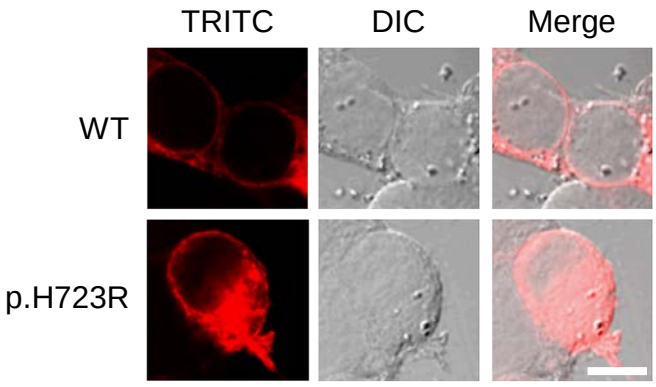


Fig. 5

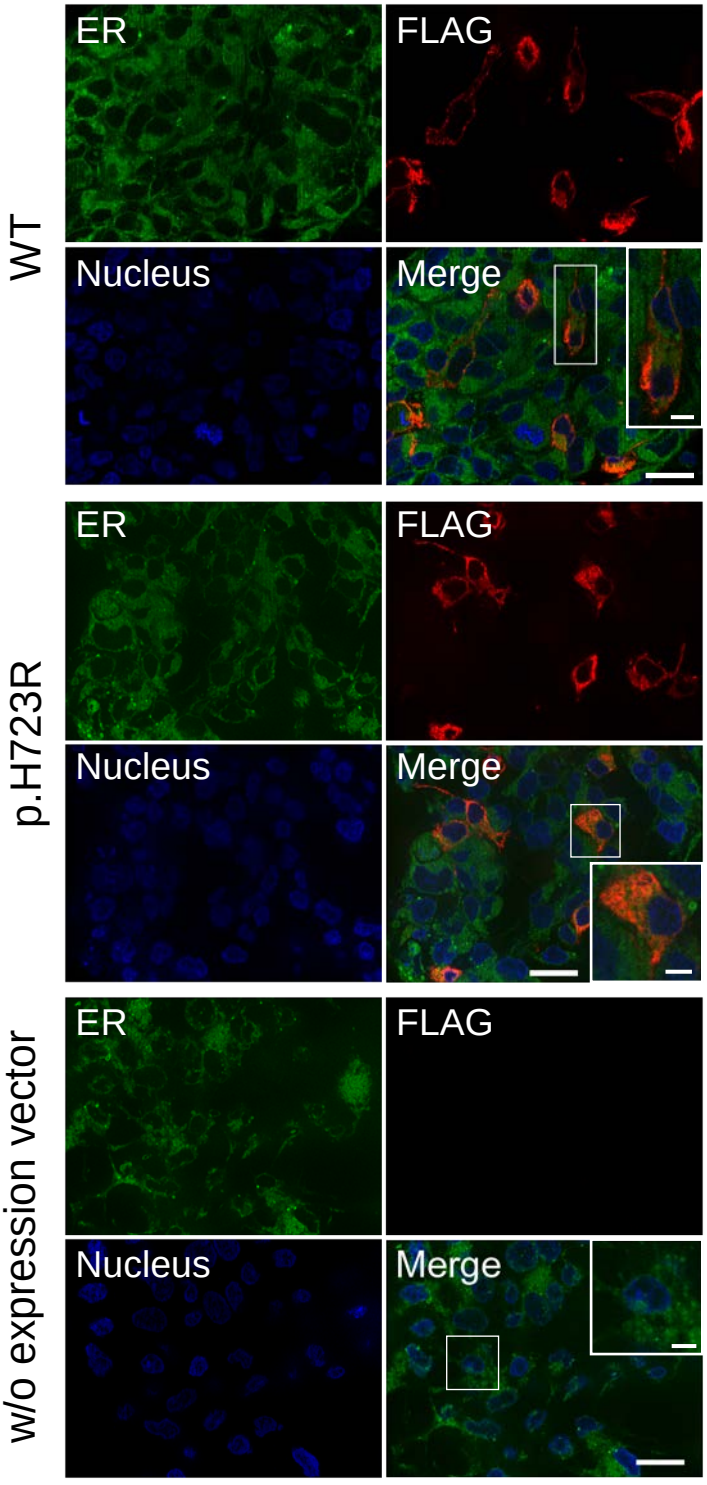

WT
ER
FLAG
Nucleus
Merge
p.H723R
ER
FLAG
Nucleus
Merge
w/o expression vector
ER
FLAG
Nucleus
Merge

Fig. 6

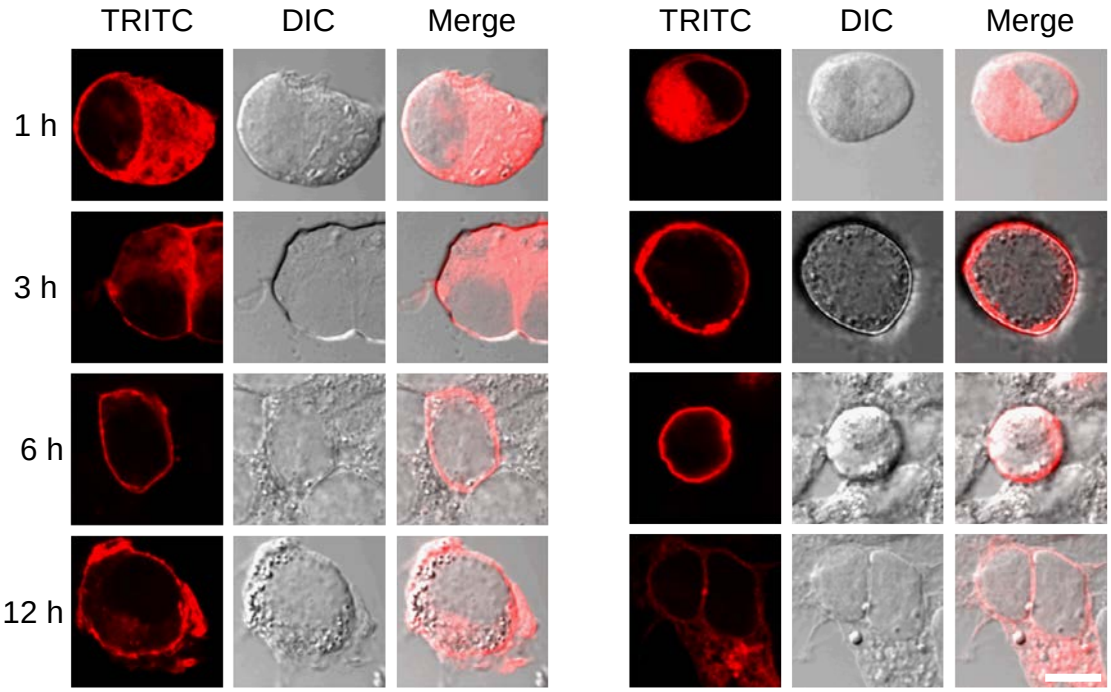

TRITC
DIC
Merge
TRITC
DIC
Merge
1 h
3 h
6 h
12 h
10 mM Salicylate
1 mM 2-Hydroxybenzyl alcohol

Fig. 7

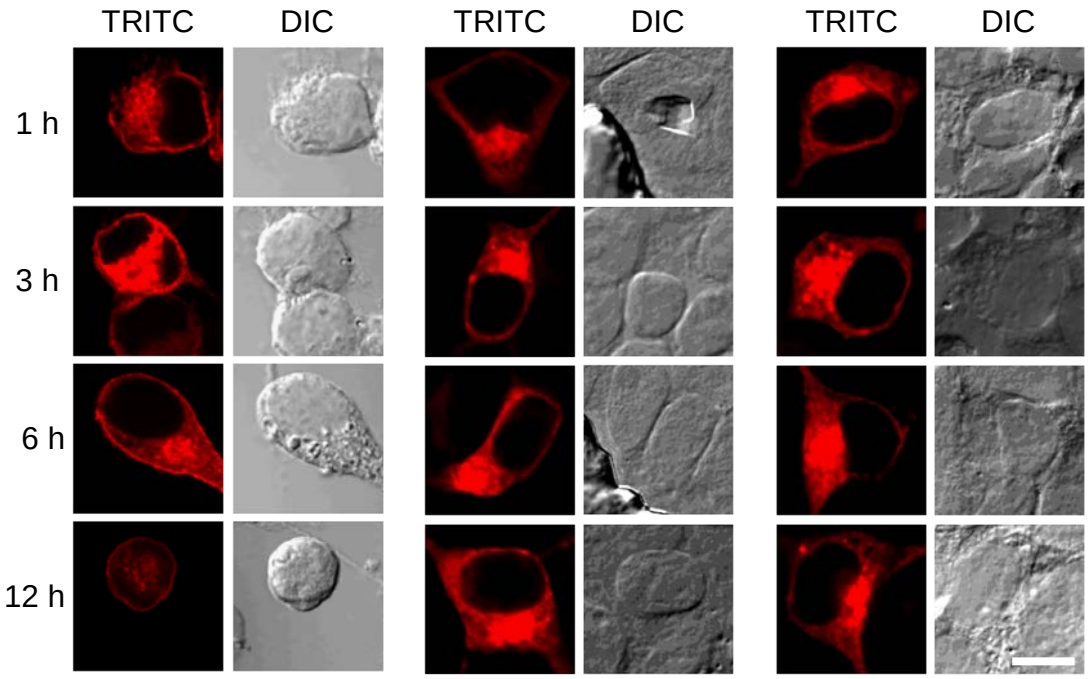

TRITC
DIC
TRITC
DIC
TRITC
DIC
1 h
3 h
6 h
12 h
1 mM
2,3-Dihydroxybenzoic acid
1 mM
2'-Hydroxyacetophenone
1 mM
Methyl salicylate

Fig. 8

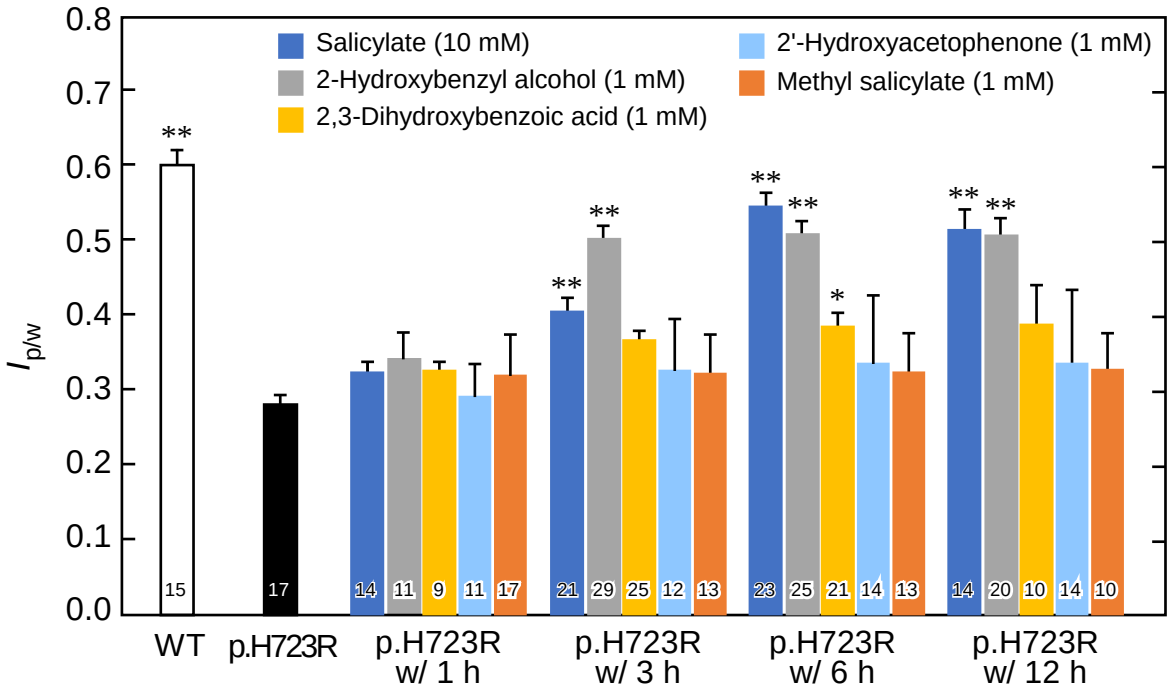
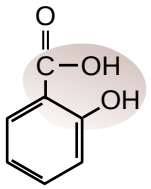
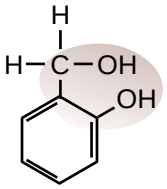

Salicylate (10 mM)
2-Hydroxybenzyl alcohol (1 mM)
2,3-Dihydroxybenzoic acid (1 mM)
2'-Hydroxyacetophenone (1 mM)
Methyl salicylate (1 mM)
$I_{p/w}$
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
WT
p.H723R
p.H723R w/ 1 h
p.H723R w/ 3 h
p.H723R w/ 6 h
p.H723R w/ 12 h

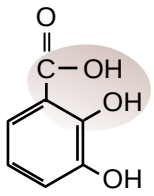
Fig. 9



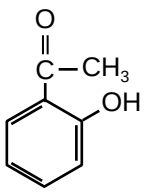
Salicylate



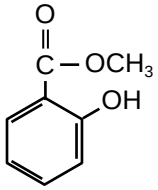
2-Hydroxybenzyl alcohol



2,3-Dihydroxybenzoic acid



2'-Hydroxyacetophenone



Methyl salicylate

Fig. 10