The Protein Translocation Defect of MCT8^{L291R} Is Rescued by Sodium Phenylbutyrate

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Keywords

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Abstract

Introduction: The monocarboxylate transporter 8 (MCT8; SLC16A2) is a specific transporter for thyroid hormones. MCT8 deficiency, formerly known as the Allan-Herndon-Dudley syndrome, is a rare genetic disease that leads to neurological impairments and muscle weakness. Current experimental treatment options rely on thyromimetic agonists that do not depend on MCT8 for cellular uptake. Another approach comes from studies with the chemical chaperone sodium phenylbutyrate (NaPB), which was able to stabilize MCT8 mutants having protein folding defects in vitro. In addition, NaPB is known as a compound that assists with plasma membrane translocation. **Objective:** The pathogenic MCT8^{L291R} leads to the same severe neurological impairments found for other MCT8-deficient patients but, unexpectedly, lacks alterations in plasma 3,3',5-triiodothyronine (T₃) levels. Here we tried to unravel the underlying mechanism of MCT8 deficiency and tested whether the pathogen-

ic MCT8^{L291R} mutant responds to NaPB treatment. Therefore, we overexpressed the mutant in Madin-Darby canine kidney cells in the human choriocarcinoma cell line JEG1 and in COS7 cells of African green monkey origin. **Results:** In our recent study we describe that the MCT8^{L291R} mutation most likely leads to a translocation defect. The pathogenic mutant is not located at the plasma membrane, but shows overlapping expression with a marker protein of the lysosome. Mutation of the corresponding amino acid in murine Mct8 (Mct8^{L223R}) displays a similar effect on cell surface expression and transport function as seen before for MCT8^{L291R}. NaPB was able to correct the translocation defect of MCT8^{L291R}/Mct8^{L223R} and restored protein function by increasing T₃ transport activity. Furthermore, we detected enhanced mRNA levels of wild-type and mutant MCT8/Mct8 after NaPB treatment. The increase in mRNA levels could be an explanation for the positive effect on protein expression and function detected for wild-type MCT8. **Conclusion:** NaPB is not only suitable for the treatment of mutations leading to misfolding and protein degradation, but also for a mutant wrongly sorted inside a cell which is otherwise functional.

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Introduction

The monocarboxylate transporter 8 (MCT8; SLC-16A2) is responsible for the transport of thyroid hormones across plasma membranes of certain cell types, including neurons [1–3]. Mutations in MCT8 lead to severe developmental and neurocognitive impairments [4, 5]. Its expression in several areas of the developing human brain [6, 7] and the symptoms of intellectual and motor disabilities found in MCT8-deficient patients suggest a pivotal role of MCT8 for proper development of the brain [nicely summarized in 8].

In addition, MCT8-deficient patients suffer from muscular hypotonia and show a typical endocrinological phenotype of high 3,3',5-triiodothyronine (T_3) levels, low 3,3',5,5'-tetraiodothyronine (thyroxine, T_4) levels, and high to normal thyroid-stimulating hormone levels. These patients are unable to walk or sit independently and do not develop any speech [9, 10]. Different mouse models have been developed to gain a better insight into the disease [11, 12]. Surprisingly, *Mct8*-deficient mice replicate the endocrinological phenotype, but do not suffer from cognitive impairments or muscular hypotonia. An additional deletion of the compensating transporter *Oatp1c1* in *Oatp1c1/Mct8* double deficient mice affords a mouse model that displays neurodevelopmental features of MCT8 deficiency [13]. The brain phenotype of these mice could be rescued by administration of the T_3 metabolite 3,3',5-triiodothyroacetic acid (Triac) [14]. Triac is a T_3 receptor agonist and is currently under investigation as a possible treatment option for MCT8-deficient patients [8, 15].

The use of chemical and pharmacological chaperones could be another treatment option for a fraction of MCT8-deficient patients. The chemical chaperone sodium phenylbutyrate (NaPB) has been successfully used for the treatment of cystic fibrosis [16, 17]. The cystic fibrosis transmembrane conductance regulator (CFTR) protein with a delPhe508 mutation undergoes rapid protein degradation of an otherwise functional protein. NaPB stabilizes mutant CFTR and thus rescues the function of the protein as a channel for chloride ions [18].

Groeneweg et al. [8] reported about at least 320 individuals in 132 families with MCT8 deficiency. Among MCT8 mutations, there are 48 mutations published that comprise insertion, deletions, or substitution of single amino acids [8]. We and others have already demonstrated that NaPB is able to stabilize and rescue the protein expression and function of seven of these MCT8 mutants in vitro [19–21]. All seven mutations are associated with

a milder phenotype of MCT8 deficiency in patients. Less severely affected MCT8-deficient patients are able to walk with some help and achieve the ability to pronounce a few words [9, 22–24]. We hypothesized that a milder phenotype correlates with decreased protein stability rather than a T_3 -specific transport defect [19, 20]. Decreased protein stability would lead to a diminished number of functional MCT8 molecules at the plasma membrane of cells, resulting in residual transport activity (absent in severely affected patients) and the milder symptoms described above.

A patient carrying a Leu291Arg mutation in MCT8 displayed an unusual phenotype. While presenting with full symptoms of MCT8 deficiency, his plasma T_3 levels were not characteristically elevated, leading to the question of the biochemical consequences of the underlying mutation [25]. We therefore chose to investigate this mutation in more depth. We overexpressed the mutant in Madin-Darby canine kidney (MDCK1) cells (canine origin), the human-derived cell line JEG1, and COS7 cells (monkey origin). The investigated MCT8^{L291R} mutant did not show decreased protein levels, but a specific translocation defect to the plasma membrane with intracellular storage of the mutant protein. Treatment of the mutant with NaPB rescued the translocation defect and led to expression of MCT8^{L291R} at the cell surface. In turn, plasma membrane expression of MCT8^{L291R} restored T_3 uptake activity in all tested cell models.

Materials and Methods

Site-Directed Mutagenesis

Cloning of human N-terminally hemagglutinin (HA)-tagged full-length MCT8 (1–613 amino acids) into pcDNA3 was performed as described in Kinne et al. [26]. The Leu291Arg mutation was introduced by site-directed mutagenesis (QuikChange Lightning; Agilent Technologies, Santa Clara, CA, USA) using the following primers: *Leu291Arg* fwd 5'-CTT TCA GCC ATC CCG CGT CAT CCT GGG CC-3', *Leu291Arg* rev 5'-GGC CCA GGA TGA CGC GGG ATG GCT GAA AG-3'. Cloning of N-terminally HA-tagged full-length murine *Mct8* (1–545 amino acids, reference sequence: NM_009197.2) from liver cDNA into pcDNA3 followed the strategy described in Kinne et al. [26]. Primers used for site-directed mutagenesis are: *Leu223Arg* fwd 5'-CCT TTC AAC CAT CAC GCG TCA TCC TGG GCC A-3', *Leu223Arg* rev 5'-TGG CCC AGG ATG ACG CGT GAT GGT TGA AAG G-3'.

Cell Culture and Stable Transfection

All cell lines were cultured in DMEM/F12 (1:1) (Gibco, Waltham, MA, USA) + 10% fetal calf serum (FCS) (Gibco) + 1% penicillin (5,000 U/mL)/streptomycin (5,000 µg/mL) (Gibco) in a humidified atmosphere at 37 °C and 5% CO₂.

Fig. 1. NaPB but not genistein restores the membrane expression and transport function of MCT8^{L291R}. **a** Endpoint assay of NaPB-treated and untreated MCT8^{L291R} compared to treated and untreated MCT8^{WT} expressed in MDCK1 cells. Treatment: 4 mM NaPB directly dissolved in medium. The cells were incubated with 2 nM ¹²⁵I-T₃ and 10 nM T₃ for 20 min. Treated and untreated mock-transfected MDCK1 cells served as negative controls and values were subtracted as background. The specific MCT8 inhibitor SC was used at 10 μ M for inhibition studies. **b** Endpoint assay of genistein-treated and untreated MCT8^{L291R} compared to treated and untreated MCT8^{WT} as described above. Treatment: 15 μ M genis-

tein dissolved in medium. DMSO-treated cells served as solvent controls. All results were obtained from three independent experiments ($n = 3$). Statistics: Two-way ANOVA with Tukey's correction for multiple comparisons. Comparison of untreated MCT8^{WT} and MCT8^{L291R}, respectively, with NaPB- and SC-treated values: #### $p < 0.0001$. Comparison of untreated/DMSO-treated MCT8 with treated and untreated MCT8^{L291R}, respectively: **** $p < 0.0001$. DMSO, dimethyl sulfoxide; MCT8, monocarboxylate transporter 8; MDCK1, Madin-Darby canine kidney; NaPB, sodium phenylbutyrate; ns, not significant; SC, silychristin; SD, standard deviation; T₃, 3,3',5-triiodothyronine.

Stable transfection of MCT8/Mct8 in MDCK1 and JEG1 cells was performed as described before [19, 27]. Several single cell clones of stably expressing MDCK1 cells were tested for NaPB responsiveness with similar results (data not shown). One of these clones was used to obtain the results depicted in the present paper. Stable transfection of JEG1 cells led to mixed cell clones.

For stable transfection of COS7 cells, the cells were seeded 1:1 in culture dishes and directly transfected with 100 ng of plasmid DNA per cm² surface area using PANfect A Transfection Reagent (PANBioTech, Aidenbach, Germany). Stably transfected cells were obtained by changing the medium to DMEM/F12 (1:1) + 10% FCS + 1% G418 (50 mg/mL) (Merck, Darmstadt, Germany) after 48 h of transfection. The treatment with G418-containing medium was continued for another week before the cells recovered in DMEM/F12 + 10% FCS + 1% penicillin/streptomycin to obtain mixed cell clones.

Chaperone Treatment and Use of Silychristin

NaPB was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and directly dissolved in medium to a final concentration of 20 mM. Genistein was obtained from Sigma Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 mM. We used the following working concentrations for treatment: 4 mM NaPB and 15 μ M genistein. A solvent control for NaPB-treated cells was not necessary; for genistein treatment, DMSO-treated cells were used as controls.

The NaPB concentrations used for the treatment of cells were chosen from the lowest concentration that showed a good response for surface expression and radioactive uptake assay of MCT8^{L291R}. Higher concentrations of NaPB also rescued mutant MCT8 expression but, in addition, lead to increased cell death, which makes the treatment with higher doses inappropriate for

additional experiments. Increased cell death is probably due to the fact that NaPB is known as an inhibitor of proliferation [28]. We tested different concentrations of genistein. None of the tested concentrations showed a positive effect on surface expression or transport function of MCT8^{L291R}.

Treatment of stably expressing MDCK1 and JEG1 cells with exogenous chaperones was performed for 48 h as described before [19]. In terms of cell viability, stably transfected COS7 cells responded more sensitively to NaPB treatment compared to MDCK1 and JEG1 cells. Thus, the duration of treatment for stably transfected COS7 cells was shortened to 24 h [21].

Silychristin (SC) was purchased from PhytoLabs (Vestenbergsgreuth, Germany) and dissolved in DMSO to a final concentration of 40 mM. 10 μ M SC was used for inhibition studies. The compound was directly given to the cells together with (non)radioactive T₃ diluted in DMEM/F12 (1:1).

Radioactive Uptake Assays

Three days before the experiments, various numbers of cells were seeded into 24-well plates (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000507439). Differences in cell numbers are explained by different growth rates of cell clones obtained during stable transfection and increased cell death during chaperone treatment. Uptake assays were performed after the treatment with chaperones for 24 h (COS7) and 48 h (MDCK1, JEG1).

The purification of ¹²⁵I-T₃ (Perkin Elmer, Waltham, MA, USA) from iodine ions was performed as described before [19, 20]. 2 nM ¹²⁵I-T₃ plus 10 nM T₃ (Sigma Aldrich) diluted in DMEM/F12 (1:1) were used for endpoint experiments (20 min) at 37 °C. Nonradioactive T₃ (Sigma Aldrich) was dissolved in 40 mM NaOH to a 20-mM stock solution. Working solutions were prepared by dilut-

Fig. 2. NaPB, but not genistein, corrects the translocation defect of MCT8^{L291R}. **a** Twenty micrograms of whole cell homogenates (upper panel) and 30 μ L of biotinylated protein (lower panel) from MDCK1 cells were analyzed by Western blotting. MCT8 was detected by anti-HA antibody. Beta-actin served as loading control. **b** Quantification of at least three Western blot analyses: NaPB (upper panel) rescued the plasma membrane expression of MCT8^{L291R} while genistein (lower panel) did not show any positive effect. Values are given as $\pm$ SD. The signal for actin was used for normalization. Statistical analyses: Two-way ANOVA with Dunnett's correction for multiple comparisons. Comparison of untreated/DMSO-treated MCT8^{WT} (darkest bars) with every other sample. ** $p < 0.01$. DMSO, dimethyl sulfoxide; HA, hemagglutinin; MCT8, monocarboxylate transporter 8; MDCK1, Madin-Darby canine kidney; NaPB, sodium phenylbutyrate; SD, standard deviation.

ing the stock solution in 20 mM NaOH. Cells were washed, lysed, and measured as described in Braun and Schweizer [19].

Western Blotting and Surface Biotinylation

Pellets from treated and untreated stably expressing MDCK1, JEG1, and COS7 cells were harvested from six-well plates and lysed

in 50 μ L homogenization buffer (250 mM sucrose; 20 mM HEPES; 1 mM EDTA in distilled H₂O; pH 7.4) with 1 mM dithiothreitol. Twenty micrograms of whole cell lysates were separated on 10% polyacrylamide gels containing sodium dodecyl sulfate, transferred on nitrocellulose membranes, and probed with antibodies against HA tag (1:1000, RRID:AB_307019) and β -actin (1:30,000,

Fig. 3. NaPB, but not genistein, restores the plasma membrane expression of MCT8^{L291R} in JEG1 and COS7 cells. **a, b** Twenty micrograms of whole cell homogenates (upper panels) and 30 μ L of biotinylated protein (lower panels) from JEG1 cells (**a**) and COS7 cells (**b**) were analyzed by Western blotting. MCT8 was detected by anti-HA antibody. Beta-actin served as loading control. **c, d** Quantification of three Western blot analyses obtained from overexpressing JEG1 cells (**c**) and overexpressing COS7 cells (**d**). NaPB (upper panels) rescued the plasma membrane expression of

MCT8^{L291R} while genistein (lower panels) did not show any positive effect. Values are given as $\pm$ SD. The signal for ACTIN/actin was used for normalization. Statistical analyses: Two-way ANOVA with Dunnett's correction for multiple comparisons. Comparison of untreated/DMSO-treated MCT8^{WT} (darkest bars) with every other sample. * $p < 0.05$, ** $p < 0.01$. DMSO, dimethyl sulfoxide; HA, hemagglutinin; MCT8, monocarboxylate transporter 8; NaPB, sodium phenylbutyrate; SD, standard deviation.

RRID:AB_262011). Secondary antibodies (RRID:AB_2313567, RRID:AB_10015289) were diluted 1:15,000. Surface biotinylation was used for the detection and purification of cell surface proteins as described before [27].

Immunohistochemistry

Stably expressing cells were seeded on uncoated coverslips and grown until they reached confluence. Cells on coverslips were fixed with ice-cold methanol for 5 min, permeabilized with 0.2%

Triton-X100 in phosphate-buffered saline (PBS) for 15 min, and blocked with 10% FCS in PBS for 1 h. Anti-HA antibody (1:400) and anti-protein disulfide isomerase (PDI) antibody (1:200, RRID:AB_2163123) were incubated at 4 °C overnight. Anti-lysosomal-associated marker protein 1 (LAMP1) antibody (1:100, RRID:AB_2296838) was incubated for 3 h at room temperature. Secondary antibodies were incubated for 1.5 h at room temperature in the dark: Alexa-Fluor-488-goat-anti-rabbit-antibody (1:800, RRID:AB_2576217), Cy3-donkey-anti-rabbit (1:800, RRID:AB_2307443), and Cy2-donkey-anti-mouse (1:200, RRID:AB_2340826). DAPI staining (1:1,000 in PBS) was performed for 30 min at room temperature. Pictures were taken with a Zeiss Axiovert 200M inverted microscope.

Quantitative Polymerase Chain Reaction

Total RNA of pellets from NaPB-treated and untreated stably expressing MDCK1, JEG1, and COS7 cells were isolated using TRIzol® (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was performed using Takyon Low ROX SYBR 2x MasterMix blue dTTP (Eurogentec, Seraing, Belgium) on a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) and the following primers for detection of human *MCT8* and *18S rRNA* for normalization: *MCT8* fwd 5'-TGC TTT CAT TGG CCT CCA TA-3', *MCT8* rev 5'-CCA GCA GAC ACC ACA CCA TT-3', *18S rRNA* fwd 5'-TTG ACG GAA GGG CAC CAG-3', and *18S rRNA* rev 5'-GCA CCA CCC ACG GAA TCG-3'. Due to sequence similarities between human *MCT8* and its murine homolog, the same primers were used for the detection of murine *Mct8*.

Statistical Analyses

Measured values (duplicates) as counts/min of radioactive uptake experiments were normalized to protein content (in mg) (online suppl. Table 1) determined for each cell clone grown in 24 wells. Calculated values as counts/min/mg for wild-type *MCT8*/*Mct8* were set to 100% ± standard deviation to combine different independent experiments in one graph. The number of replicates is given in the figure legends.

Western blot analyses were performed three times with samples from three independent experiments. One representative result is given in the figures. ImageJ was used for the quantification of Western blots. qPCR analyses were performed with samples harvested from three independent experiments. The expression values of wild-type *MCT8*/*MCT8* were set to 1 ± standard deviation to combine different independent experiments in one graph.

GraphPad Prism 6 was used for the generation of graphs and for statistical analysis. The statistical test is given in the figure legends. Error bars are smaller than symbols if not visible.

Fig. 4. Mutant *MCT8*^{L291R} shows an overlapping expression pattern with the lysosomal marker LAMP1 in JEG1 cells. **a** Immunohistochemical staining confirms the translocation defect of untreated *MCT8*^{L291R} in MDCK1, JEG1, and COS7 cells. In COS7 cells *MCT8*^{L291R} was hard to see at the plasma membrane, although T₃ transport activity was clearly present. NaPB reduced the intracellular storage of mutant *MCT8*^{L291R}. Anti-HA-antibody was used to stain wild-type and mutant *MCT8* green. **b** Immunohisto-

Results

NaPB Increases the Transport Function of MCT8^{L291R}

MCT8^{L291R} expressed in MDCK1 cells was not able to transport T₃ (Fig. 1a). The treatment of cells expressing *MCT8*^{L291R} and *MCT8*^{WT} with NaPB significantly increased T₃ transport activity. SC is known as a specific inhibitor of *MCT8* [29]. SC significantly inhibited T₃ uptake of NaPB-treated and untreated wild-type *MCT8* as well as NaPB-treated *Leu291Arg* mutant (Fig. 1a). We used SC to prove that the NaPB-mediated increase in T₃ uptake exclusively depends on the rescue of *MCT8*^{L291R} and not on upregulation of another endogenous thyroid hormone transporter.

In addition to NaPB, we tested whether the soy isoflavone genistein could positively affect the activity of the *Leu291Arg* mutant, which we have observed for other *MCT8* mutants with protein folding defects before [19, 20]. Genistein was not able to increase the T₃ uptake neither for *MCT8*^{L291R} nor for *MCT8*^{WT} (Fig. 1b). The results obtained for genistein treatment point towards the fact that the underlying mechanism of the mutant's pathogenicity is not associated with a protein folding defect.

We found similar effects after NaPB and genistein treatment when *MCT8*^{L291R} was expressed in JEG1 and COS7 cells (online suppl. Fig. 1).

NaPB Corrects the Translocation Defect of MCT8^{L291R}

Surface biotinylation experiments provide information about the expression of a protein at the cell surface. *MCT8*^{L291R} showed a strong expression in whole cell lysates of MDCK1 cells (Fig. 2a, upper panels). However, the expression of the mutant at the plasma membrane was reduced (Fig. 2a, lower panels). NaPB, but not genistein, rescued the expression of *MCT8*^{L291R} at the plasma membrane while *MCT8*^{WT} expression remained unaltered (Fig. 2a, lower panels). Quantification of protein expression levels and statistical analyses (Fig. 2b) supported the results found in Figure 2a. Similar results for *MCT8*^{L291R} were obtained when testing overexpressing JEG1 and COS7 cells (Fig. 3). *MCT8*^{L291R} expressed in both cell lines led to a reduction of the protein at the plasma membrane

chemical costaining of *MCT8*^{L291R} (red) expressed in JEG1 cells as well as the ER marker PDI and the lysosomal marker LAMP1 (both green). Scale bars, 50 µm. ER, endoplasmic reticulum; HA, hemagglutinin; LAMP1, lysosomal-associated marker protein 1; *MCT8*, monocarboxylate transporter 8; MDCK1, Madin-Darby canine kidney; NaPB, sodium phenylbutyrate; PDI, protein disulfide isomerase; T₃, 3,3',5-triiodothyronine.

(For figure see next page.)

Fig. 5. Murine Mct8^{L223R} replicates the translocation defect found for human MCT8^{L291R}. **a** Transport activity of Mct8^{L223R}-MDCK1 cells treated with or without 4 mM NaPB compared to wild-type Mct8. The cells were incubated with 2 nM [¹²⁵I]-T₃ and 10 nM T₃ for 20 min. Treated and untreated mock-transfected MDCK1 cells served as negative controls and were subtracted as background. *n* = 3. Statistics: Two-way ANOVA with Tukey's correction for multiple comparisons was used to compare treated with untreated wild-type Mct8 and Mct8^{L223R}. #### *p* < 0.0001. In addition, untreated wild-type Mct8 was compared with untreated and treated Mct8^{L223R}. **** *p* < 0.0001. **b** Twenty micrograms of whole cell homogenates (left panel) and 30 μL of biotinylated protein (right panel) from treated and untreated MDCK1 cells stably expressing wild-type Mct8 and Mct8^{L223R} were analyzed by Western blotting.

Mct8 was detected by anti-HA antibody. Beta-actin served as loading control. **c** Quantification of three Western blot analyses showing that murine Mct8^{L223R} replicates the translocation defect found for human MCT8^{L291R}. NaPB rescued the plasma membrane expression of mutant Mct8^{L223R}. Values are given as ±SD. The signal for actin was used for normalization. Statistical analyses: Two-way ANOVA with Dunnett's correction for multiple comparisons. Comparison of untreated Mct8^{WT} (darkest bars) with every other sample. **p* < 0.05, ***p* < 0.01. **d** Immunohistochemical staining of Mct8^{WT} and untreated and NaPB-treated Mct8^{L223R}. Anti-HA antibody was used to detect Mct8 expression. Scale bar, 50 μm. HA, hemagglutinin; MCT8, monocarboxylate transporter 8; MDCK1, Madin-Darby canine kidney; NaPB, sodium phenylbutyrate; SD, standard deviation; T₃, 3,3',5-triiodothyronine.

that could be restored by NaPB but not genistein. Unlike the unaltered MCT8^{WT} expression at the cell surface of MDCK1 and JEG1 cells after NaPB treatment, the expression of wild-type MCT8 in COS7 cells was significantly increased (Fig. 3d).

Immunohistochemical staining of MCT8^{L291R} expressed in MDCK1, JEG1, and COS7 cells compared to MCT8^{WT} confirmed that the expression pattern of MCT8^{L291R} was restricted to intracellular compartments (Fig. 4a). Thus, MCT8^{L291R} displayed a translocation defect, which led to intracellular storage of the mutant protein. Treatment with NaPB partly helped the mutant protein to translocate to the plasma membrane (Fig. 4a).

In order to investigate whether MCT8^{L291R} is trapped in the endoplasmic reticulum (ER), we stained stably expressing JEG1 cells with the ER marker PDI. We were not able to detect an overlapping expression of MCT8^{L291R} with PDI in the ER (Fig. 4b, left panel). However, we could identify a colocalization of the mutant with LAMP1 (Fig. 4b, right panel). Thus, in overexpressing JEG1 cells MCT8^{L291R} is routed to the lysosome when it is not properly transported to the plasma membrane. Unfortunately, antibodies used as markers for the ER and the lysosome did not work in MDCK1 or COS7 cells.

Fig. 6. NaPB increases the mRNA levels of wild-type and mutant *MCT8/Mct8* expressed in MDCK1 cells. **a** Human *MCT8* (*SLC16A2*). **b** Murine *Mct8* (*Slc16a2*). mRNA levels were measured by qPCR and normalized to *18S rRNA*. Values are given as $\pm$ SD. Statistics: One-way ANOVA (Tukey's correction for multiple comparison). ** $p < 0.01$, **** $p < 0.0001$. MCT8, monocarboxylate transporter 8; MDCK1, Madin-Darby canine kidney; NaPB, sodium phenylbutyrate; ns, not significant; qPCR, quantitative polymerase chain reaction; SD, standard deviation.

Expression of Murine Mct8^{L223R} Reveals the Same Phenotype Found for Human MCT8^{L291R}

The corresponding amino acid of Leu291 in murine Mct8 is Leu223. Leu223Arg stably expressing MDCK1 cells showed the same T₃ transport defect (Fig. 5a) as seen before for human MCT8^{L291R} (Fig. 1a). Similar to MCT8^{L291R}, Mct8^{L223R} shows a strong expression in whole cell lysates. However, surface biotinylation experiments showed that the expression of Mct8^{L223R} at the cell surface is reduced compared to wild-type Mct8 (Fig. 5b). Quantification of Western blot analyses confirmed these findings (Fig. 5c). Immunohistochemical stainings of Mct8^{L223R} compared to wild-type Mct8 showed a similar cytosolic distribution pattern of the mutant as seen before

for MCT8^{L291R}-MDCK1 cells (Fig. 4a, 5d). NaPB was able to restore the T₃ transport function of Mct8^{L223R} by rescuing the translocation defect and increasing the amount of functional Mct8^{L223R} molecules at the plasma membrane (Fig. 5). NaPB also induced the expression and function of Mct8^{WT}.

NaPB Increases mRNA Expression in MCT8/Mct8 Overexpressing Cells

The expression of MCT8^{WT}/Mct8^{WT} in all tested cell models (MDCK1, JEG1, and COS7 cells) showed a significant increase in protein function after NaPB treatment (Fig. 1a; online suppl. Fig. 1; [19]). Thus, NaPB did not only affect the expression and function of mutated proteins with protein folding or translocation defects, but also increased wild-type protein expression. In order to find an explanation, we measured *MCT8/Mct8* mRNA levels by qPCR and found a significant three- to four-fold increase of MCT8^{WT}/Mct8^{WT} after NaPB treatment in all tested overexpressing cell models (Fig. 6; online suppl. Fig. 2). The significant increase in mRNA levels after NaPB treatment was not only observed for MCT8^{WT}/Mct8^{WT}, but also for MCT8^{L291R}/Mct8^{L223R} mutant mRNA (Fig. 6; online suppl. Fig. 2).

Discussion

A promising pharmacological treatment for Allan-Herndon-Dudley syndrome (AHDS) that is currently under clinical investigation is Triac, an MCT8-independent T₃ receptor agonist that enters a cell independent of MCT8 [14, 30–33]. The Triac Trial I successfully reported the reduction of the thyrotoxic effect of high T₃ and the safety of Triac administration in MCT8-deficient patients [15]. The Triac Trial II (NCT02396459) is going to elucidate the effect of Triac on neurocognitive development [8]. Sobetirome and its prodrug Sob-AM2 are additional thyromimetic agents that do not depend on MCT8 for cellular entry. These compounds showed promising effects in a mouse model of MCT8 deficiency [34].

An alternative with a more restricted spectrum of responsive MCT8 mutations is NaPB. NaPB has been shown to rescue mutant membrane proteins and has been used for the treatment of cystic fibrosis [16, 17] and hereditary cholestasis [35, 36]. Its safety for children has been demonstrated as a treatment for urea cycle defects where it acts as a scavenger for excess nitrogen [37].

We have already reported that NaPB (and to a lesser extent genistein) can rescue pathogenic MCT8 mutants

with protein folding defects in vitro which lead to a milder phenotype of the disease in humans [19, 20]. Here we expand our previous work and identified a pathogenic MCT8 mutation responding to NaPB, but not genistein treatment, that does not have a protein folding but a trafficking defect.

To date, there is one MCT8 patient published carrying a Leu to Arg substitution at amino acid position 291 leading to a severe form of AHDS [25]. As first symptoms the patient displayed poor head control and reduced muscular tone. The parents reported feeding difficulties, developmental delay, and no achievement of independent sitting or walking. Thus, MCT8 function was likely severely compromised in the nervous system. Unexpectedly, the authors reported normal T_3 levels found in this patient [25]. Although the authors published the results of just one measurement, the unchanged T_3 values attracted our attention and we started to investigate the Leu291Arg mutant in more detail. We tried to unravel the biochemical consequence of the mutation and found that it disturbed normal trafficking of the mutated protein to the plasma membrane in all tested cell lines. Detailed investigation of the distribution pattern of MCT8^{L291R} expressed in human-derived JEG1 cells revealed that the mutant is able to escape from the ER but is routed to the lysosomal compartment. Our homology models [38] predict that Leu291 is located at the end of transmembrane helix four facing the intracellular environment. In general, an unstable plasma membrane protein is sent for lysosomal degradation after its recognition by the cytosolic C-terminus of HSC70-interacting protein (CHIP) [39]. If the mutation of Leu291 to Arg creates a new CHIP recognition site and, thus, would lead to lysosomal degradation of a functional protein remains uncertain.

Another hypothesis includes misfolding of MCT8^{L291R} mutant protein followed by aggregation. In general, misfolded proteins are sent for ER-associated degradation and finally degraded in the cytosol by the ubiquitin-proteasome system (UPS) [40]. Some pathogenic proteins are UPS-resistant and form aggregates in the cytosol. Protein aggregates are usually eliminated by the autophagolysosomal system [41]. If MCT8^{L291R} belongs to an UPS-resistant protein, this could explain its presence in the lysosome.

NaPB is not only known as a chemical chaperone rescuing proteins with folding defect, but also as a compound helping mutant proteins with translocation to the plasma membrane [35, 36]. Here, we tested the Leu291Arg mutant for NaPB responsiveness. We found that the translocation defect could be corrected by pharmacolog-

ical doses of NaPB in all tested cell lines, suggesting a cell type-independent action of NaPB on MCT8^{L291R}. Genistein had no positive effect on translocation processes. NaPB-corrected surface expression led to a quantitative restoration of MCT8^{L291R} T_3 transport function. Thus, the mutation does not affect T_3 binding or transport but the plasma membrane expression of an otherwise fully functional protein.

Several mechanisms of NaPB action have already been reported, including downregulation of endogenous chaperones [42] and attenuation of ER stress by interacting with the unfolded protein response machinery [43, 44]. The molecular mechanism behind NaPB-mediated rescue of membrane proteins that are stuck in the ER has recently been investigated. NaPB competes with p24 proteins for binding to COPII coat proteins, leading to an attenuation of ER retention of resident and misfolded proteins [45]. However, the mechanism by which NaPB is able to avoid lysosomal sorting remains to be elucidated.

In order to investigate the effect of NaPB on the mutant in vivo, the use of a transgenic mouse model carrying a Leu223Arg mutation would be desirable. The mouse model would also help to figure out whether mice with Mct8^{L223R} mutation replicate unexpected T_3 levels similar to those found in the patient. The murine mutant showed the same translocation defect as the human mutant when expressed in MDCK1 cells. In addition, Mct8^{L223R} displayed an equal responsiveness towards NaPB as observed before for MCT8^{L291R} and would therefore represent a suitable model to investigate NaPB action in vivo.

In addition to the positive effects on membrane translocation, NaPB is also known as an inhibitor of histone deacetylases [37]. Acetylation of histones facilitates transcription by influencing chromatin remodeling and alters gene expression. Thus, inhibition of histone deacetylation should increase mRNA levels of certain genes. Indeed, an upregulation of mRNA levels after NaPB treatment has already been reported [21].

Similar to the results found in Groeneweg et al. [21], we also observed an upregulation of MCT8/Mct8 mRNA levels in our overexpressing cell models. The increase in mRNA could be an explanation for the increased expression and transport function of human wild-type MCT8 expressed in JEG1 and COS7 cells as well as murine wild-type Mct8 expressed in MDCK1 cells after NaPB treatment. Another explanation for the increase in MCT8 mRNA could be found in its stabilization. We have already tested other MCT8 mutants for mRNA stability and detected an increased half-life of wild-type and

mutant *MCT8* mRNA after NaPB treatment (data not shown). From an energetic point of view the cell would benefit from a longer half-life since it spends a lot of energy on transcription of *MCT8* mRNA with its large (>100 kb) first intron.

Groeneweg et al. [21] reported that NaPB does not induce *MCT8* mRNA in patient-derived fibroblasts. The authors hypothesized that the induction of *MCT8* mRNA depends on its promoter. In patient-derived fibroblasts, *MCT8* is under control of a native promoter, while *MCT8* expression depends on an artificial promoter in overexpressing cells. Further experiments would be necessary to elucidate whether there is an NaPB-mediated effect on *MCT8* mRNA level in other patient-derived cell lines, e.g., differentiated induced pluripotent stem cells.

Expression of human *MCT8*^{WT} in COS7 cells displayed an increase in protein expression in cell lysates and at the plasma membrane after NaPB treatment that was not detectable in MDCK1 cells. Groeneweg et al. [21] demonstrated that a low plasmid concentration for transient transfection of COS1 cells displayed the strongest NaPB effect. A low plasmid concentration leads to lower protein content in the cell. Conversely, we hypothesize that MDCK1 cells expressing *MCT8*^{WT} with high protein abundance are not able to significantly increase their protein expression after NaPB treatment because of reaching their general protein expression capacity.

Unlike the expression of *MCT8*^{WT} expressed in MDCK1 cells, murine *Mct8*^{WT} was significantly increased after NaPB treatment in MDCK1 cells, most likely due to lower transfection efficiency and protein abundance. In general, the expression of membrane proteins is a highly regulated process. Brown et al. [46] reported that only 20–25% of newly synthesized wild-type CFTR protein pass the quality control system of a cell and reach its plas-

ma membrane. Thus, we hypothesize that NaPB can promote the expression of *Mct8*^{WT} (and *MCT8*^{WT} expressed in JEG1 and COS7 cells) in our overexpressing cell systems by the increase of mRNA levels and the mechanisms mentioned above [42–44].

In conclusion, the understanding of the underlying mechanism of a given pathogenic *MCT8* mutation could be helpful to find a specific personalized treatment for AHDS patients. NaPB is an approved drug for the treatment of children and could be a new tool for the treatment of patients carrying mutations that cause protein folding and also the newly discovered translocation defects.

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Conflict of Interest Statement

No competing financial interests exist.

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Author Contributions

D. Braun designed and performed the experiments. D. Braun and U. Schweizer wrote the manuscript.

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