Efficient activation of pathogenic ΔPhe501 mutation in monocarboxylate transporter 8 by chemical and pharmacological chaperones*

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Monocarboxylate transporter 8 (MCT8) is a thyroid hormone transmembrane transporter expressed in many cell types, including neurons. Mutations which inactivate transport activity of MCT8 cause severe X-linked psychomotor retardation in male patients, a syndrome originally described as the Allan-Herndon-Dudley syndrome. Treatment options currently explored focus on finding thyroid hormone-like compounds which bypass MCT8 and enter cells through different transporters. Since MCT8 is a multipass transmembrane protein, some pathogenic mutations affect membrane trafficking while potentially retaining some transporter activity. We explore here the effects of chemical and pharmacological chaperones on expression and transport activity of the MCT8 mutant ΔPhe501. Dimethylsulfoxide, 4-phenylbutyric acid as well as its sodium salt, and the isoflavone genistein increase T_3 uptake into MDCK1 cells stably transfected with mutant MCT8-ΔPhe501. We show that ΔPhe501 represents a temperature sensitive mutant protein which is stabilized by the proteasome inhibitor MG132. 4-phenylbutyrate has been used to stabilize ΔPhe508 mutant CFTR protein and is in clinical use in patients with urea cycle defects. Genistein is enriched in soy and available as a nutritional supplement. It is effective in stabilizing MCT8-ΔPhe501 at 100 nM concentration. Expression of the L471P mutant is increased in response to phenylbutyrate, but T_3-uptake activity is not induced supporting the notion that the chaperone specifically increases membrane expression. Our findings suggest that certain pathogenic MCT8 mutants may be responsive to (co-)treatment with readily available compounds which increase endogenous protein function.

Mutations in monocarboxylate transporter 8 (MCT8, *SLC16A2*) lead to severe psychomotor retardation in male patients (1, 2). The Allan-Herndon-Dudley syndrome (AHDS; OMIM 300 523) was first described in 1944 as an inherited mental retardation syndrome (3) and the pathogenic mutations affecting the *MCT8* gene were identified 60 years later (4). MCT8 is a transmembrane transport protein mediating import and export of thyroid hormones across the plasma membrane (5). Both thyroid hormones, 3,3′,5-triiodothyronine (T_3) and 3,3′,5,5′-tetraiodothyronine (T_4, thyroxine), as well as 3,3′,5′-triiodothyronine (rT_3) and 3,3′-diiodothyronine (3,3′-T_2) are substrates of human MCT8 (6). All pathogenic MCT8 mutants described so far impair transporter function (7–10). Pathogenic mutations in *MCT8* include missense, as well as nonsense, splice site, and indel mutations, arguing that the disorder is caused by loss-of-function of *MCT8*.

MCT8 is expressed in neurons, astrocytes, and brain endothelial cells (11–15). Our current understanding is that MCT8 mutations reduce the import of T_3 and T_4 into the brain and into neurons and thus impair neural development (12, 16, 17). Although other thyroid hormone transporter proteins are expressed in the brain (18), mutations in MCT8 apparently limit thyroid hormone function during a crucial developmental period and beyond. Patients carrying certain mutations show a less severe clinical phenotype (AHDS) (4, 19). When tested in vitro, some of the respective mutant MCT8 proteins display signifi-

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Abbreviations: [1]MCT8, monocarboxylate transporter 8; MDCK1, Madin-Darby canine kidney cells; T_3, 3,3′,5-triiodo-L-thyronine; 3,3′-T_2, 3,3′-diiodo-L-thyronine; T_4, 3,3′,5,5′-tetraiodo-L-thyronine, thyroxine; DMSO – dimethylsulfoxide; 4PBA – 4-phenylbutyric.acid; NaPB – sodium 4-phenylbutyrate; TMAO – trimethylamine-N-oxide; NaTC – sodium taurocholate.

cant residual transport activity in at least some cell systems (8, 19). It may thus be possible that even incomplete rescue of brain thyroid hormone levels may improve the patients' phenotypes. Similarly, 3,5-diiodothyropropionic acid (DITPA) (20) and 3′,3,5-triiodothyroacetic acid (TRIAC) are explored as therapeutic options for patients (NCT02060474). The therapeutic principle is the same for both approaches: both compounds act as agonists at the nuclear T_3-receptor and bypass the MCT8 transporter. Animal experiments have suggested that both DITPA and TRIAC can exert thyromimetic actions in the brain (21–23). Both approaches should be applicable to patients carrying any type of inactivating mutation in MCT8.

A number of missense mutants show T_3-uptake activity when expressed in certain cell types suggesting that the corresponding mutations do not primarily affect the catalytic mechanism of MCT8, but the correct insertion of MCT8 into the plasma membrane (8). Visser et al, described the ΔPhe501 mutation in MCT8 which leads to a milder phenotype in the patient and retains significant thyroid hormone uptake activity in JEG3 cells and patient-derived fibroblasts (19). We have reasoned that such mutants may be targets for chemical or pharmacological chaperones and tested several candidate mutants (8). Chaperones are proteins facilitating protein folding by preventing aggregation (24). Chemical chaperones are small compounds which likewise support correct protein folding by altering cellular solvation conditions or interaction with protein quality control (QC) mechanisms, while pharmacological chaperones are small compounds which enhance protein folding by stabilizing the target protein during folding transitions (25, 26). Often, but not always, pharmacological chaperones mimic the physiological substrate of an enzyme. Tafamidis, a pharmacological chaperone, stabilizes mutant transthyretin by occupying the normally empty T_4 sites in the tetramer and thus prevents aggregation of monomers (27). Mutations in the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) cause CF, a severe disorder which is characterized by defective chloride export across epithelia (28). One of the most frequent mutations in CFTR, ΔPhe508, impairs membrane insertion of the mutant protein and leads to degradation (29). Several chemical chaperones are effective in vitro in rescuing ΔPhe508 CFTR (30), these include dimethylsulfoxide (DMSO), glycerol, and 4-phenylbutyric acid (4PBA). Some of these compounds, like 4PBA or its sodium salt are also effective in vivo (31–33) and based on the concept, effective pharmacological chaperones have been developed for CFTR (34). Since sodium 4-phenylbutyrate (NaPB) is approved for and in clinical use as therapy for patients with urea cycle defects, it may represent a real treatment option for at least some patients with mutations in MCT8.

Here we tested whether the ΔPhe501 mutation in MCT8 responds to treatment with chemical chaperones. We show that membrane expression and thyroid hormone uptake activity of ΔPhe501 MCT8 can be rescued in vitro by the chemical chaperones DMSO, 4PBA, NaPB and the pharmacological chaperone genistein in a dose-dependent fashion. The effects are apparent in stably transfected MDCK1 cells and in *Xenopus* oocytes injected with cRNA. Expression of the L471P mutant of MCT8, which is also unstable, can be increased by chemical chaperones as well, but the mutant protein is not functional as transporter. This result suggests that the observations on ΔPhe501 are a result of stabilization of the mutant protein.

Materials and Methods

Site-directed mutagenesis and sequencing analysis

Mutations of ΔPhe501 was introduced into human MCT8 using QuikChange™ Lightning Site-Directed Mutagenesis Kit (Stratagene) with the following primers: ΔPhe501 fwd 5′cctttgcgatggcttcatcaccatcatggccc 3′, ΔPhe501 rev 5′ gggccatgatggtgatgaagccatcgcaaagg 3′.

RNA from MDCK1 cells stably expressing mutant protein was purified with Trizol (Invitrogen) according to the manufacturer's protocol. 1 μg of RNA was reverse transcribed in cDNA with iScript cDNA synthesis kit (BioRad). The following primers were used to amplify mutant MCT8 cDNA with Kapa Hifi polymerase (PeqLab): MCT8 fwd 5′ caccatgtaccctatgatgtc 3′ (binds to the HA-tag) and MCT8 rev 5′ gattggttcctcagggttg 3′. The same primers together with MCT8–600bp fwd 5′ agttccaagcagcatgggtc 3′ and MCT8–1200bp fwd 5′ ttctggctcagctcaggaag 3′ were used for sequencing. Sanger sequencing followed according to standard methods.

Chemicals

DMSO, NaPB, TMAO, NaTC and MG132 were purchased from Sigma-Aldrich. 4PBA was obtained from Merck. Genistein was a gift from Branca Jurjevic. 4PBA, NaPB, TMAO, NaTC and DMSO were directly dissolved in medium. Genistein and MG132 were dissolved in DMSO to a final concentration of 100 mM and 10 mM, respectively.

Stable transfection of MDCK1 cells

MDCK1 cells were cultured in DMEM/F12 (1:1) (GIBCO) + 10% fetal calf serum (FCS) (GIBCO). For stable transfection MDCK1 cells were seeded in 6 cm plates and grown until they reached 40%–50% confluence. 6 μg of plasmid DNA were incubated with PolyFect transfection reagent (Qiagen) and added to the cells. After 48 hours of transfection the medium was changed to DMEM/F12 (1:1) + 10% FCS + 0.5% G418 (50 mg/ml) (Merck). Within two to three weeks most of the cells died and only single cells survived which formed colonies. The colonies were scraped from the plate and transferred to a 24 well plate to allow the cells to recover. When reaching 70%–80% conflu-

ence, the cells were split into 6 wells plates and T25 flasks. Cells from the flasks were frozen in DMEM/F12 (1:1) + 10% FCS + 1% penicillin (5000U/ml)/streptomycin (5000 μg/ml) + 10% DMSO (Sigma-Aldrich). Protein was harvested from the 6 well plates in homogenization buffer (250 mM saccharose, 20 mM Hepes, 1 mM EDTA in dH_2O, pH 7.4) with 1 mM DTT and screened by Western blotting. Three ΔPhe501 clones were selected and frozen, and two clones were used for subsequent experiments.

Chaperone treatment

Stably transfected MDCK1 cells were cultured in DMEM/F12 (1:1) + 10% FCS + 1% penicillin/streptomycin. Cells were seeded in 6 well plates and grown until they reached 30%–40% confluence. Chaperone treatment started for 48 hours with a change of the medium after 24 hours. Since DMSO showed an increase of ΔPhe501 expression in MDCK1 cells, DMSO served as solvent control for genistein and MG132. After treatment, the cells were washed and harvested for western blotting in 100μl homogenization buffer with 1 mM DTT. 25 μg whole protein lysate were separated on SDS gels.

Surface biotinylation and Western blotting

Surface biotinylation was used for the detection and purification of cell surface proteins as previously described (35). Briefly, equal amounts of 5 μg of biotinylated protein were separated on SDS gels, transferred onto nitrocellulose membranes, and probed with an MCT8 antibody (Atlas, Stockholm, Sweden). An antibody directed against β-actin (Rockland, Gilbertsville, USA) was used for loading control. For Western Blotting of *Xenopus laevis* oocytes, 3 × 3 oocytes were lysed in 90 μl homogenization buffer with 1 mM DTT and treated with ultrasound. 15 μl were separated on SDS gels, transferred onto nitrocellulose membranes and probes as described above.

Radioactive uptake experiments

Three days before the experiments, 55 000–120 000 cells per well were seeded into 12-well plates. The chaperone treatment started one day after seeding as described above.

^{125}I-T_3 and ^{125}I-T_4 (Perkin Elmer), respectively, was purified from iodide ions by adsorption chromatography and finally resuspended in DMEM/F12 (1:1) without serum (6). Nonradioactive iodothyronines were dissolved in 20 mM NaOH. For time course and endpoint assays, stably transfected cell lines were incubated with 2–10 nM ^{125}I-T_3 or 10 nM ^{125}I-T_4, respectively. When using 2 nM ^{125}I-T_3, 10 nM nonradioactiv T_3 was added. Cells were incubated for 1 to 30 minutes (time course) or 20 minutes (endpoint assay), respectively, at 37°C. After a double wash with ice cold PBS, cells were lysed in 40 mM NaOH, and radioactivity of the lysate was measured in a gamma counter (LB2111 Multi Crystal, Berthold Technologies). Radioactivity associated with empty vector-transfected cells was subtracted as background. Measurements of IC_{50} values, K_m values and mode of inhibition were performed as described elsewhere (35, 36) with the exception of the genistein experiment where incubation time was 10 minutes.

Uptake measurements in *Xenopus oocytes*

In vitro transcription of 5 μg plasmid DNA were performed using the mMessage mMachine® T7 Kit (Life Technologies, Paisley, UK) following the manufacture's protocol. *Xenopus laevis* oocyte preparation and cRNA injection were accomplished by EcoCyte Bioscience (Castrop-Rauxel, Germany). Oocytes were injected with 30 ng cRNA. Injected oocytes were kept in Barth's buffer (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$ x 7 H_2O, 0.66 mM $NaCO_3$, 0.75 mM $CaCl_2$ x 2 H_2O, 50 mM Hepes in dH_2O, pH 7.4 + 10 mg/l gentamycin) for additional three days before performing uptake measurements at room temperature.

Radioactive compounds were dissolved in Barth's buffer with the following concentrations: 10 nM ^{125}I-T_3. For time course assays, oocytes were incubated with tracer for 1 to 30 minutes, washed with ice cold PBS and lysed with 40 mM NaOH. Absorbed radioactivity of each oocyte were measured using a gamma counter (LB2111 Multi Crystal, Berthold Technologies). H_2O injected oocytes served as negative controls.

Immunofluorescence staining

Cover slips were coated with DMEM/F12 (1:1) + 5% FCS + 1% penicillin/streptomycin + 10 mM HEPES (AppliChem) + 1% Collagen G (4 mg/ml) (Biochrom) at 4°C over night. Cells were seeded on coated cover slips in DMEM/F12 (1:1) + 10% FCS + 1% penicillin/streptomycin and grown until the reached confluence. 4% paraformaldehyde (AppliChem) in phosphate buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, pH 7.4) was used for fixation of the cells (10 minutes, room temperature). Cover slips were washed twice with 1x PBS before incubating the cells with 1x PBS + 0.2% Triton X-100 (AppliChem) for 15 minutes. The cells were blocked in 1x PBS + 0.2% Triton X-100 + 2% BSA (AppliChem) for one hour at room temperature. Antibodies against MCT8 and zona occludens 1 (ZO-1) were diluted in blocking solution and used for incubating the cells at 4°C over night in the dark. After washing the cells with 1x PBS, the cells were blocked again for 30 minutes. Monoclonal Cy2 (mouse anti rabbit) were diluted in 1x PBS + 0.2% Triton and given to the cells for 30 minutes in the dark.

Microscopy

Stained sections were imaged at 63x (Plan-Aprochromat 63x/1.4 Oil, Carl Zeiss Microscopy GmbH) with a laser scanning confocal microscope (LSM 510, Carl Zeiss Microscopy GmbH), using the LSM software package. Channels were scanned separately to avoid signal contamination. Cy2 was excited using the 488 nm line of an argon ion laser. The emission signal was trun-

Table 1.

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
human MCT8	amino acids 52–155	MCT8	ATLAS, Stockholm, Swe, HPA003353	rabbit	"1:500"
against β-actin		actin	Rockland, Gilbertsville, USA	rabbit	"1:2000"
ZO1		ZO1-Alxexa594	Invitrogen	mouse	10 μg/mL
ant-rabitt		anti-rabitt Cy2	Dianova	mouse	"1:800"

cated by an optical bandpass filter (500–550 nm). Alexa 594 was excited at 543 nm via a helium-neon laser. The emission signal was truncated by a 560 nm low-pass filter.

Results

Low expression and activity of ΔPhe501 MCT8 in MDCK1 cells and *Xenopus* oocytes can be increased by the chemical chaperone DMSO

The ΔPhe501 MCT8 mutant ($MCT8^{\Delta F501}$) expresses at low levels in stably transfected MDCK1 cells and $^{125}I\text{-}T_3$ uptake activity does not exceed empty vector control cells (Figure 1A). Since several attempts to find clones with higher $MCT8^{\Delta F501}$ expression failed, we generated capped cRNA encoding $MCT8^{\Delta F501}$ and injected it into *Xenopus* oocytes. After three days of incubation, T_3 uptake was assessed. $MCT8^{\Delta F501}$ did not exceed the background radioactivity values associated with H_2O-injected control oocytes (Figure 1B). Wild-type MCT8 showed the expected expression and activity in both systems. In order to exclude the possibility that additional MCT8 mutations occurred in our cell clones, the presence of ΔPhe501 was verified by sequencing of cDNA from the MDCK1 clone (Figure 1C). The full MCT8 sequence is given in Supplemental Figure 1.

$MCT8^{\Delta F501}$-expressing MDCK1 cells were incubated with increasing concentrations of DMSO. Western blot analysis of cellular protein revealed a dose-dependent, over thirty-fold increase of $MCT8^{\Delta F501}$ (Figure 2A). Cell surface exposed $MCT8^{\Delta F501}$ accumulated at about 50% of $MCT8^{WT}$ levels when the cells were incubated with 1.5% DMSO for two days (Figure 2B). Immunocytochemistry revealed that DMSO treatment increased $MCT8^{\Delta Phe501}$ expression in MDCK1 cells in a pattern resembling $MCT8^{WT}$ (Figure 2C). Under these conditions, significant T_3-uptake activity was apparent in $MCT8^{\Delta F501}$ cells, albeit still lower than in cells expressing $MCT8^{WT}$ (Figure 2D).

According to our homology model, Phe501 is located in the middle of transmembrane helix 9 in a region which is expected to form a kink within the helix (Figure 3A). Amino acid Asp498, which is essential for activity is lo-

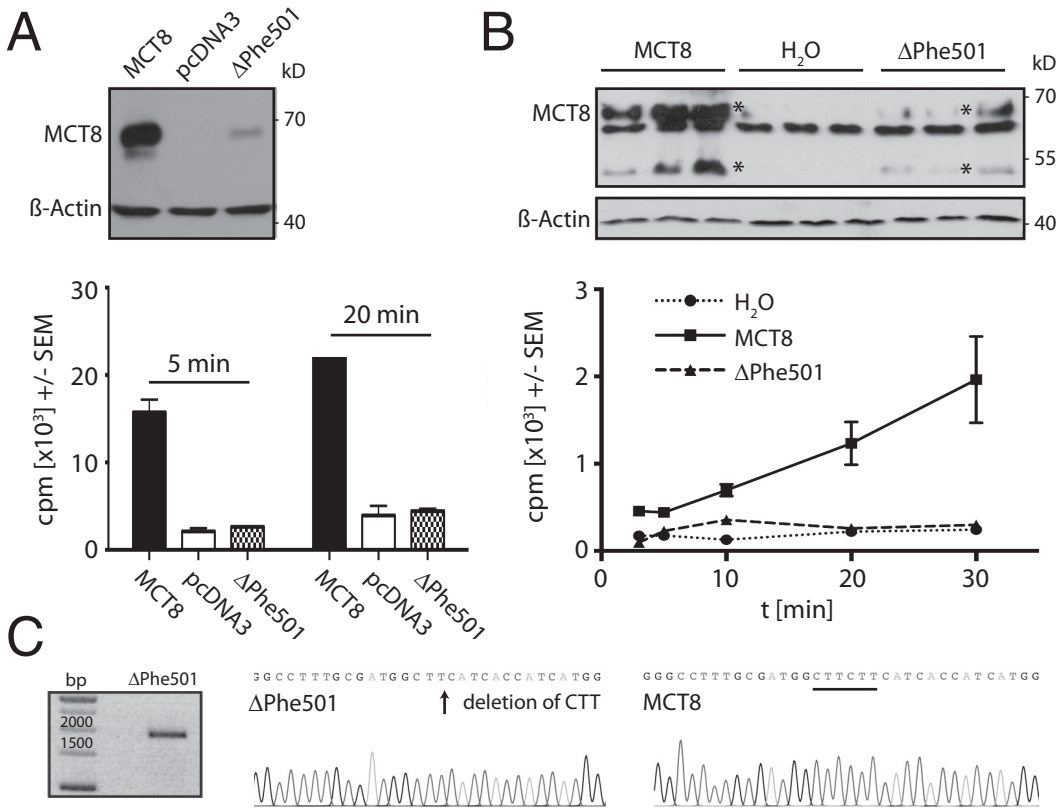

Figure 1. $MCT8^{\Delta F501}$ expresses weakly in MDCK1 cells and *Xenopus* oocytes. A) Reduced expression of $MCT8^{\Delta F501}$ in stably transfected MDCK1 cells. Western blot against MCT8 in whole cell homogenates. β-actin served as loading control. pcDNA3: empty vector transfected cells. *Lower panel*: End point assay of 2 nM $^{125}I\text{-}T_3$ uptake into cells. B) Reduced expression of $MCT8^{\Delta F501}$ in *Xenopus* oocytes. 30 ng cRNA was capped and injected into oocytes. H_2O was injected as negative control, $MCT8^{WT}$ served as positive control. Note the presence of an unspecific band slightly smaller than MCT8. *Lower panel*: Time course of 10 nM $^{125}I\text{-}T_3$ uptake into *Xenopus* oocytes. The mutant did not exceed the background of mock-injected cells. C) Sequence analysis of amplified cDNA, which was obtained from $MCT8^{\Delta F501}$ expressing MDCK1 cells by reverse transcription, shows the deletion of the triplett CTT.

cated nearby (6, 37). The deletion of one amino acid within an α-helix is expected to alter the helix register and would twist the C-terminal part of the helix by –110°, if the helix were not interrupted anyway. The amino acid deletion could destabilize the protein during membrane insertion or interfere with correct folding of the protein.

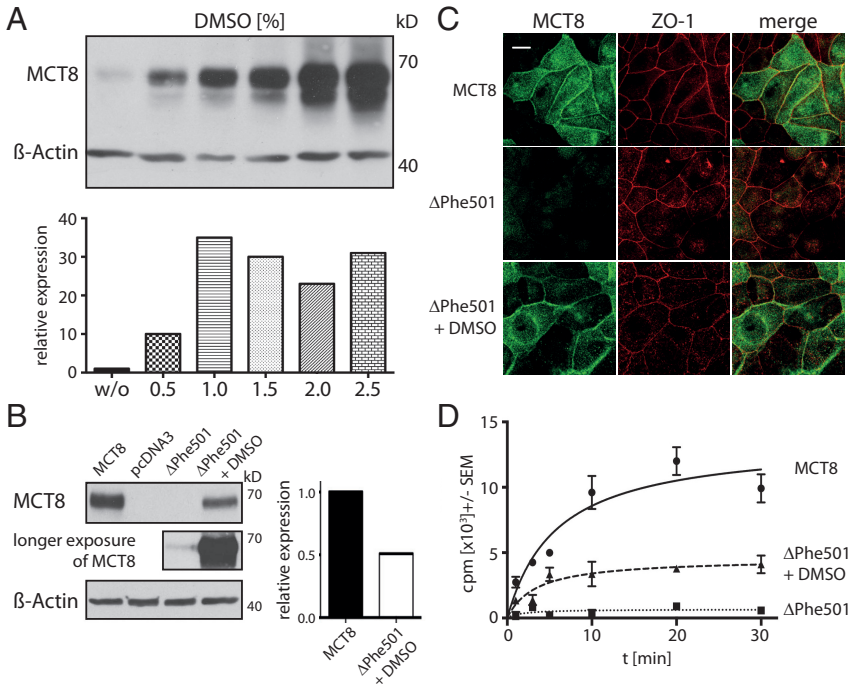

Figure 2. DMSO treatment rescues MCT8$^{\Delta F501}$ expression and function in MDCK1 cells. A) DMSO treatment of MCT8$^{\Delta F501}$ cells for two days dose-dependently increased MCT8 expression in whole cell homogenates. ß-actin served as loading control. B) Cell surface expression of MCT8^{WT}, empty vector, MCT8 $^{\Delta F501}$, and MCT8$^{\Delta F501}$ in the presence of 1.5% DMSO. *Right*: densitometric evaluation. C) Immunofluorescence staining of MDCK1 cells stably expressing MCT8^{WT}, MCT8 $^{\Delta F501}$, and DMSO (1.5% for two days) treated MCT8$^{\Delta F501}$. Zona occludens 1 (ZO-1) served as a marker for plasma membrane expression. Scale bar: 20 μM D) Time course of 10 nM ^{125}I-T$_3$ uptake into MCT8^{WT}, MCT8$^{\Delta F501}$ and 1.5% DMSO-treated MCT8$^{\Delta F501}$ cells.

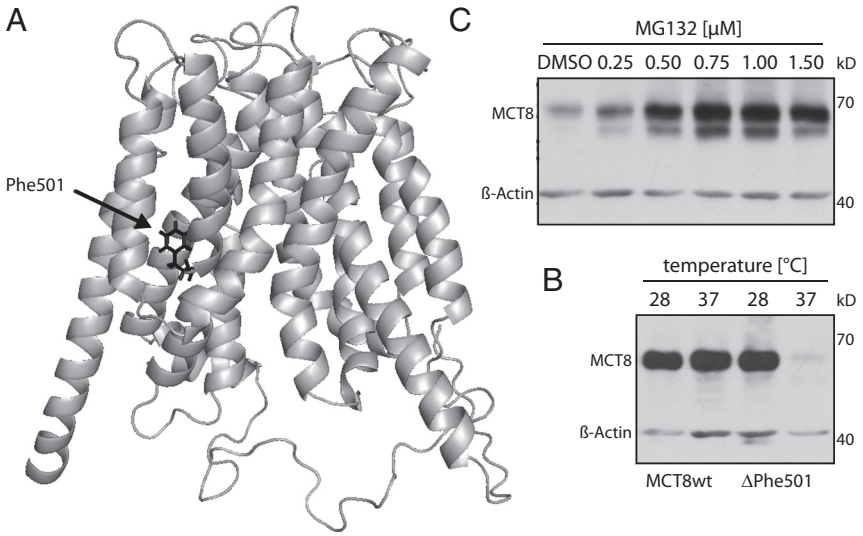

Figure 3. The MCT8$^{\Delta F501}$ mutant is a temperature-sensitive protein which is degraded via the proteasome. A) Model of MCT8 with Phe501 (black) indicated in helix 9. B) Mutant MCT8 is expressed in stably transfected MDCK1 cells and cultured at 28°C and 37°C for two to four days to probe for temperature sensitivity. Western blot of total protein, β-actin served as loading control. C) Mutant MCT8 is stabilized by incubation with the proteasome inhibitor MG132. Western blot of total protein, β-actin served as loading control.

We speculated that the ΔPhe501 mutant may be temperature-sensitive. Incubation of cells at 28°C indeed rescued MCT8$^{\Delta F501}$ expression, while 37°C clearly represents a non-permissive temperature (Figure 3B). Degradation of unstable membrane proteins is expected to proceed via the ERAD (endoplasmic reticulum associated degradation) pathway and to be sensitive to proteasome inhibition. We therefore exposed MCT8$^{\Delta F501}$ cells to increasing concentrations of the proteasome inhibitor MG132 and found that MCT8$^{\Delta F501}$ protein accumulated in the presence of inhibitor in a dose-dependent fashion (Figure 3C).

Exposure to 4-phenylbutyric acid or its sodium salt increased MCT8$^{\Delta F501}$ expression and activity in MDCK1 cells

Cells stably expressing MCT8$^{\Delta F501}$ were exposed for two days to 4PBA or NaPB in the low millimolar range. These treatments significantly and dose-dependently increased MCT8 protein abundance in the cells (Figure 4A). 4 mM 4PBA and 4 mM NaBP were as effectively increasing MCT8$^{\Delta F501}$ protein as 2% DMSO (Figure 4B). Measurements of T$_3$-uptake over time demonstrated substantial rescue of MCT8$^{\Delta F501}$ transport activity when cultured in the presence of chemical chaperones (Figure 4C). Even if the velocity of ^{125}I-T$_3$ uptake was reduced, T$_3$ uptake reached about 50% of wildtype MCT8 values at 20 minutes incubation time. In order to exclude the possibility that the chemical chaperones act as well on the wildtype MCT8, we incubated MDCK1 cells expressing MCT8 at the indicated concentrations with NaPB, DMSO, and genistein (see below) for two days and assessed pro-

tein abundance by Western blot. All compounds did not increase MCT8 expression in transfected cells (Figure 4D). Next we excluded that the compounds increased ^{125}I-T_3 uptake by any MCT8-independent mechanism and assessed T_3 transport in vector-transfected controls and MCT8^{WT}-transfected cells (Figure 4E). These experiments showed that the chemical chaperones specifically increase expression and activity of MCT8$^{\Delta F501}$.

Genistein increased MCT8$^{\Delta F501}$ expression and T_3-uptake activity

The effects of the chemical chaperones trimethylamine-N-oxide (TMAO) and sodium taurocholate (NaTC) were marginal when compared to DMSO and 4PBA. In contrast, the isoflavone genistein, a soy product, significantly enhanced expression of MCT8$^{\Delta F501}$ (Figure 5A). Genistein was recently described as an inhibitor of deiodinase 1 with an IC_{50} value of 3 μM (38) suggesting steric similarity between genistein and thyroid hormones. Genistein treatment increased MCT8$^{\Delta F501}$ expression to almost 80% of wildtype, ie, similar to the effcts of DMSO and NaPB. TMAO and NaTC showed less impressive effects (Figure 5B). Accordingly, only genistein was able to substantially increase T_3 uptake into MCT8$^{\Delta F501}$ expressing MDCK1 cells (Figure 5C).

If genistein can mimic thyroid hormones, it may not only act as a pharmacological chaperone, but at the same time may as well inhibit MCT8. We therefore exposed cells expressing MCT8^{WT} to ^{125}I-T_3 and competed its uptake with increasing concentrations of genistein (Figure 6A). An IC_{50} value around 14 μM shows that the isoflavone genistein also acts an inhibitor of MCT8 activity. Kinetic analysis revealed a noncompetitive mode of inhibition of MCT8^{WT} (Figure 6B,C). Interestingly, 100 nM genistein, ie, far below the IC_{50} for inhibition, already maximally increased MCT8$^{\Delta F501}$ expression (Figure 6D). Ten- and hundred-fold higher genistein concentration did not further increase MCT8 expression (Figure 6D) or T_3 uptake (Figure 6E).

We then asked whether coincubation of the chemical chaperone NaPB and the pharmacological chaperone genistein exerts an additive effect on uptake of thyroid hormones into MCT8$^{\Delta F501}$ MDCK1 cells. While T_3 uptake was increased by each NaPB and genistein, coincubation did not result in any further increase (Figure 6F).

Kinetic characterization of MCT8$^{\Delta F501}$

How good a transporter is MCT8$^{\Delta F501}$ once it is rescued by chemical chaperones and arrived at the plasma membrane? To find this out, we have determined K_M values for T_3 uptake in MDCK1 cells stably expressing mutant MCT8, but incubated with chemical chaperones in order to stabilize the mutant protein (Figure 7). In the presence of 2% DMSO and 4 mM NaPB, v_{max} is still greatly reduced, while K_M is not significantly different from MCT8^{WT}. K_M(WT)= 4.8 ± 0.9 μM,

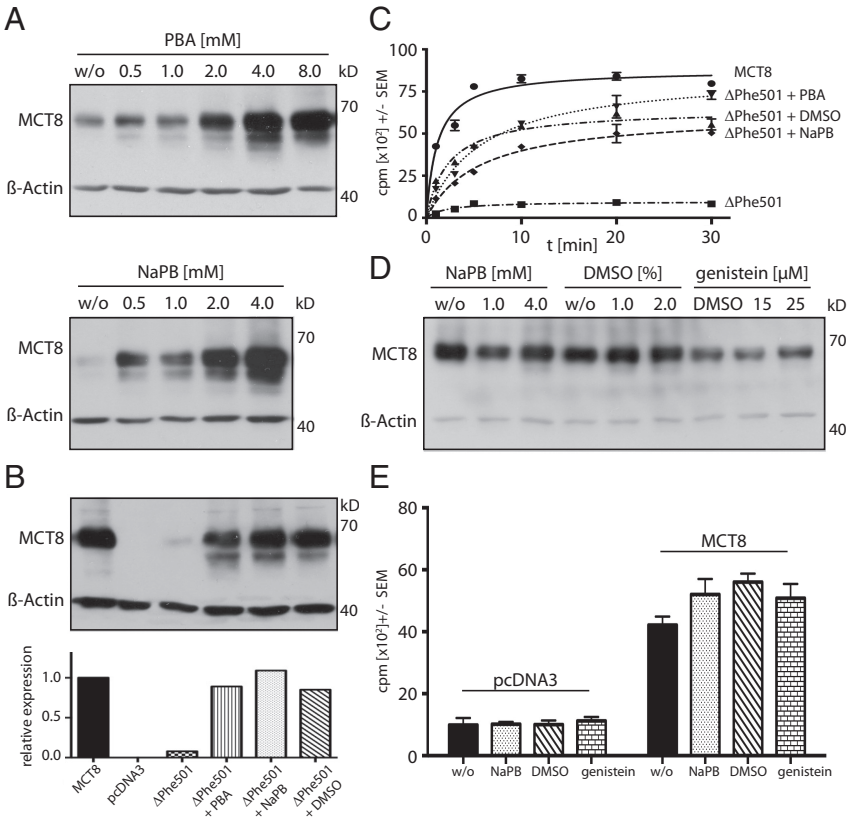

Figure 4. Treatment of MCT8$^{\Delta F501}$ cells with 4PBA or NaPB for two days substantially rescues protein expression and T_3-uptake activity. A) 4PBA and NaPB dose-dependently increase expression of MCT8$^{\Delta F501}$ in whole cell homogenates. ß-actin served as loading control. B) Expression as of MCT8$^{\Delta F501}$ in MDCK1 cells is increased by 4 mM 4PBA, 4 mM NaBP, and 2% DMSO compared to wildtype MCT8. Empty vector-transfected cells served as negative control. C) Time course of 2 nM ^{125}I-T_3 uptake into MCT8$^{\Delta F501}$ MDCK1 cells is stimulated by 4 mM 4PBA and NaPB. 2% DMSO served as positive control along with MCT8^{WT}. The background of empty-vector transfected cells was subtracted from saturation curves. D) The treatment of MDCK1 cells stably expressing MCT8^{WT} with NaPB, DMSO and genistein for two days did not affect the expression of the wildtype protein. Western blot of total protein, ß-actin served as loading control. E) The treatment of MCT8^{WT} and empty vector-transfected cells (pcDNA3) with 4 mM NaPB, 2% DMSO or 15 μM genistein for two days marginally increase the uptake of 2 nM ^{125}I-T_3. Endpoint assay: t = 20 minutes.

K_M(ΔPhe501,DMSO)= 4.4 ± 0.6 μM, and K_M(ΔPhe501,NaPB)= 2.2 ± 0.8 μM. In another experiment, K_M(WT) = 3.9 ± 0.6 μM vs. K_M(ΔPhe501, genistein) = 4.3 ± 2.4 μM were determined. The low activity in presence of 15 μM genistein may be related to its inhibitory activity. In summary, the low activity of ΔPhe501 can be attributed to its low expression at the plasma membrane.

Then, we asked whether chemical chaperone treatment of $MCT8^{\Delta F501}$ also increases T_4 uptake, since all uptake assays presented so far were done with T_3. Incubation with 2% DMSO, 4 mM 4PBA, or 15 μM genistein increased ^{125}I-T_4 uptake into MDCK1 cells in a similar fashion as T_3 uptake (Figure 8).

Chemical chaperone effects on $MCT8^{L471P}$ expression and T_3-uptake activity

We hypothesized that chemical chaperones can only increase T_3-uptake, if the MCT8 mutant is transport-competent, but has difficulty reaching the plasma membrane or is unstable in the membrane. We therefore selected the mutant L471P, which is known to respond to chemical chaperones (8), but reported inactive as transporter, and repeated our studies with this mutant. Three clones of stably transfected MDCK1-L471P ($MCT8^{L471P}$) cells were selected which displayed reduced, but significant protein expression (Figure 9A). Since the clones responded very similar, we show here only data from one clone. Incubation of L471P cells with PBA (Figure 9B) or NaPB (Figure 9C) dose-dependently increased expression of mutant MCT8. L471P was less responsive to genistein (Figure 9D). ^{125}I-T_3 uptake was not increased in the L471P mutant when exposed to 4 mM PBA, 4 mM NaPB, or 15 μM genistein, although surface biotinylation experiments clearly increase membrane translocation (Figure 9E). These experiments show that the chemical chaperones cannot activate a transport-incompetent MCT8 mutant. The apparent greater effect of genistein on ΔPhe501 expression suggests that genistein interacts better with ΔPhe501 than with L471P arguing against effects unrelated to MCT8.

Discussion

Pharmacological treatment of MCT8 deficiency

The only known function of MCT8 is mediating thyroid hormone transport across the plasma membrane. Apart from its role in thyroid hormone release from the gland (39, 40), MCT8 catalyzes uptake of thyroid hormones into target cells. Failure of MCT8 function thus deprives dependent cells of their appropriate hormonal signaling. Approaches aimed at normalizing peripheral thyroid function tests, did not positively affect the neurological phenotype (41). For this reason more recent therapeutic strategies have focused on thyromimetic substances which enter target cells independent of MCT8. DITPA treatment was explored in *Mct8*-deficent mice (42). Several patients have received DITPA over more than 40 months (20). The authors reported that DITPA does not cure the neurological deficits of the patients, but it improves their thyroid function tests, prevents development of seizures, and is well tolerated. Accordingly, this treatment continues on compassionate grounds. Treatment of cells and *Mct8*-deficient mice with TRIAC and TETRAC appeared promising (21, 22). Edward Visser initiated a clinical trial (NCT02060474) exploring the treatment of *MCT8*-deficient patients with TRIAC, which, in con-

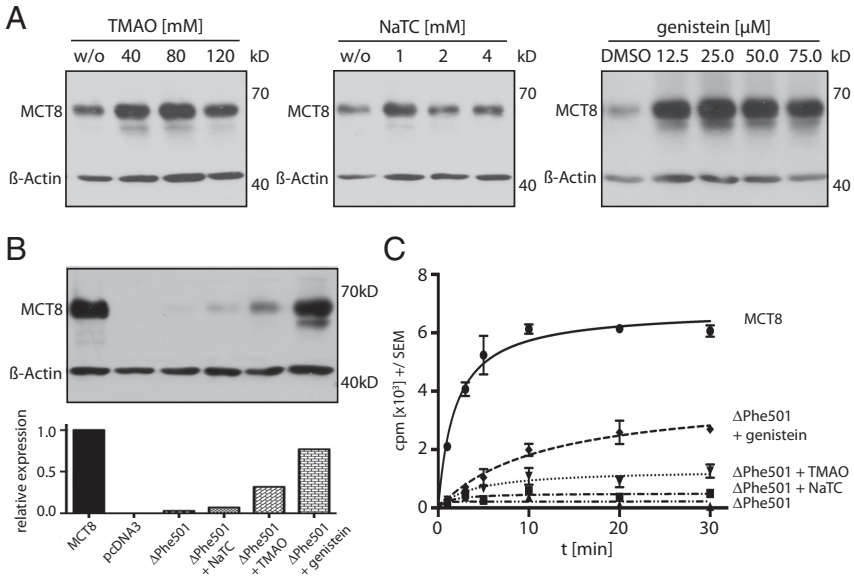

Figure 5. Genistein acts as a pharmacological chaperone on $MCT8^{\Delta F501}$ and is more effective than TMAO and NaTC. A) TMAO and NaTC only marginally increase $MCT8^{\Delta F501}$ expression, while genistein treatment for two days stimulates MCT8 expression in the μM range. Western blot of total protein, β-actin served as loading control. B) Expression of $MCT8^{\Delta F501}$ in whole cell homogenates of MDCK1 cells is increased by 15 μM genistein. Western Blot. ß-actin served as loading control. Lower panel: densitometry. C) Time course of 2 nM ^{125}I-T_3 uptake into $MCT8^{\Delta F501}$ -expressing MDCK1 cells. Genistein clearly increases T_3 uptake activity, while TMAO (40 mM) and NaTC (1 mM) are only slightly above background of untreated cells. $MCT8^{WT}$ served as positive control. The background of empty-vector transfected cells was subtracted from saturation curves.

trast to DITPA, is an approved drug in Europe for TSH suppression and prescribed for patients with resistance to thyroid hormone or differentiated thyroid cancer. Histological analyses of brains from a fetus and a child with MCT8-deficiency indicate early neurodevelopmental phenotypes which may not be readily corrected by postnatal treatment (16). In *Mct8*-knockout mice, DITPA can enter the fetus (23).

Molecular pathology and chaperone treatment as potential therapeutic option

Viewed from a molecular perspective, MCT8 is an integral membrane protein. Knowledge of the molecular pathology of MCT8 may also open ways to rescue function (8, 10, 19). Dysfunction of mutant MCT8 may be caused by mutations which directly block transport activity, because substrate interaction or conformational changes are impaired. Another class of mutant MCT8 proteins may, in principle, be active, but fail to correctly insert into the membrane. A paradigm for this case is the ΔPhe508 mutant of CFTR, which causes defective chloride secretion across certain epithelia. Due to its relatively high prevalence in Northern European populations, much research has been directed at activating $CFTR^{\Delta F508}$ (30, 32, 34). We have explored whether $MCT8^{\Delta F501}$ is a potential target for chemical chaperones. We found that $MCT8^{\Delta F501}$ is a temperature-sensitive (ie, unstable) mutant which is degraded via the proteasomal pathway. Its expression and activity is very low in MDCK1 cells and *Xenopus* oocytes, but was reported significant in JEG cells (19). This observation and our earlier observation of cell type-dependent activities of certain MCT8 mutants led us to conclude that some mutant proteins are potentially active transporters, but are removed in certain cells by membrane protein QC pathways. Such pathways can be partly overcome, at least in vitro, by altering the properties of the solvent, eg, with DMSO, glycerol, or TMAO. DMSO treatment massively increased MCT8-ΔPhe501 expression in MDCK1 cells. More importantly, T_3 and T_4 transport activity was also significantly increased showing that the $MCT8^{\Delta F501}$ mutant protein retains significant transport activity, but is degraded because of its limited stability. Kinetic analyses revealed that rescued $MCT8^{\Delta F501}$ has K_M values close to the WT protein. Chemical chaperones may act via changing solvent properties or by modulation of protein chaperones (24, 43), which help target proteins to fold properly and shield them from degradation until folding is completed (or terminally failed). We have not yet studied the mechanism how $MCT8^{\Delta F501}$ is stabilized by each of the chemical chaperones used, but the positive response to proteasome inhibition suggests that mutant protein is initially made and then degraded. Chemical chaperones may modulate protein chaperone expression or compo-

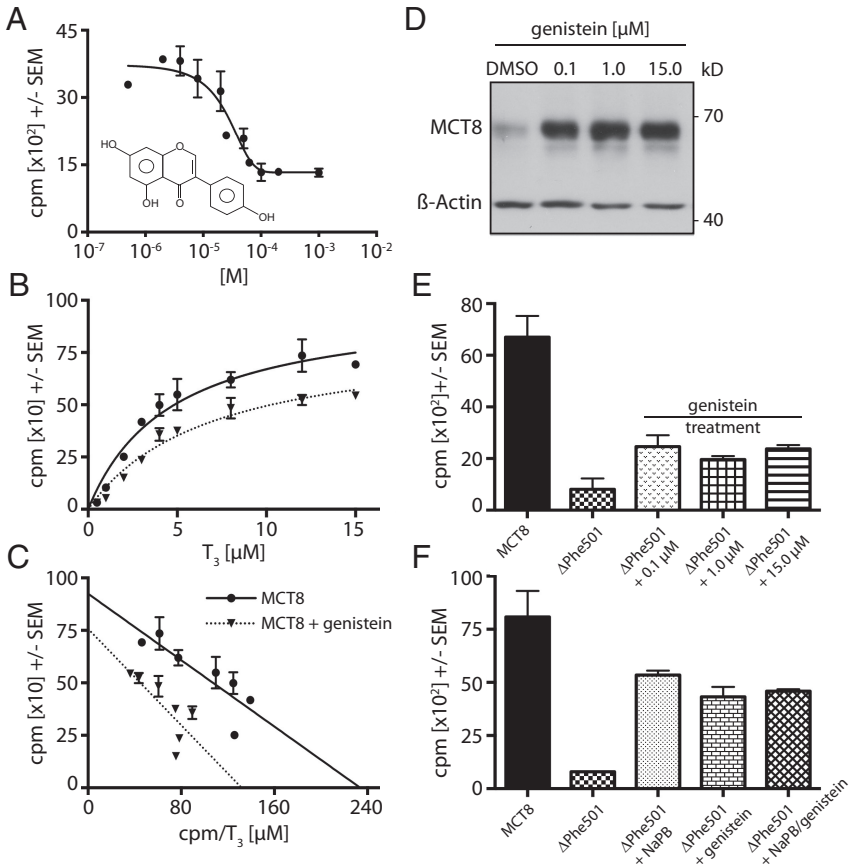

Figure 6. Genistein acts as an inhibitor of MCT8 at high concentrations, but is a pharmacological chaperone at low concentrations. A) T_3 uptake into MDCK-1 cells is inhibited by genistein with an IC_{50} of 14 μM. B) Mode of genistein inhibition – Michaelis-Menten kinetics. The uptake of increasing concentrations of T_3 into MCT8 expressing MDCK1 cells were performed in the presence and absence of 25 μM genistein. C) The transformation of the Michelis-Menten kinetic to an Eadie-Hofstee plot showed a noncompetitive mode of inhibition mediated by genistein. D) Genistein increases expression of $MCT^{\Delta F501}$ at 100 nM concentration when treated for two days. Western blot of total protein, ß-actin served as loading control. E) Genistein increases uptake activity of $MCT8^{\Delta F501}$ at concentrations far below the IC_{50} value of inhibition. F) No additive effect of chemical and pharmacological chaperones on thyroid hormone uptake. 2 nM ^{125}I- T_3 uptake in the presence of 4 mM NaPB, 15 μM genistein, and both (two day treatment). Wildtype MCT8 and empty vector-transfected cells served as positive and negative controls, respectively. Endpoint assay: t = 20 minutes.

nents of the ERAD pathway could be down-regulated in our MDCK1 cells. Genistein is known to alter lipid membrane properties and thus may affect the membrane protein QC machinery (44).

At present, it appeared most important to us to extend our studies to compounds which could be used for the treatment of patients. NaPB is a drug approved for the use in patients with ammonia cycle defects. It has also been used to increase the activity of $CFTR^{\Delta F508}$ in patients and ameliorate endoplasmic reticulum stress in mice (26). In addition, NaPB is described as a histone deacetylase inhibitor (32). Our data presented here show that NaPB significantly and dose-dependently increases $MCT8^{\Delta F501}$ expression and activity in MDCK1 cells. Again, we did not explore whether NaPB reduced protein chaperones as reported (43). We rather asked whether a pharmacological chaperone, ie, a substance which forms a complex with the target protein and thereby stabilizes its conformation, could be identified for $MCT8^{\Delta F501}$. Genistein may share some steric similarity with thyroid hormones, as it is known to bind to transthyretin (45), and recent data show that genistein inhibits deiodinase 1 in vitro (38). While an endocrine disrupting potential of genistein acting on the thyroid axis was suspected, recent assessment of the evidence suggests it a rather safe compound (46). We show here that genistein acts as an inhibitor of MCT8-mediated thyroid hormone transport, but at an IC_{50} value of 14 μM. It is thus not clear whether genistein acts as a chemical chaperone altering lipid-protein interactions or as a pharmacological chaperone interacting with a folding intermediate of $MCT8^{\Delta F501}$. In any case, its chaperone activity appears at lower concentrations than its inhibitory activity. Clearly, other mechanisms of action could be possible. The efficacy of DMSO and NaPB to increase $MCT8^{L471P}$ expression and membrane translocation, but inefficiency to increase transport activity underscores that the effect depends on the type of mutation. At the moment, it may be more important for patients to identify compounds which can be used to treat humans (NaPB and genistein) than studying underlying molecular mechanisms in vitro. Thus, the question is which patients may benefit from such treatments? We speculate that these are patients carrying mutations in MCT8 which retain some residual activity in any cell system. The applicability of chemical and pharmacological chaperones may thus be limited to a only small number of patients with certain mutations, but given the severity of the disease and the burden on those caring for the patients, any improvement should be important even for a small number of patients. We have not yet undertaken a systematic study among all known pathogenic MCT8 mutations to identify those which respond to chaperone treatment, in particular to NaPB and genistein, but this is certainly an important next step.

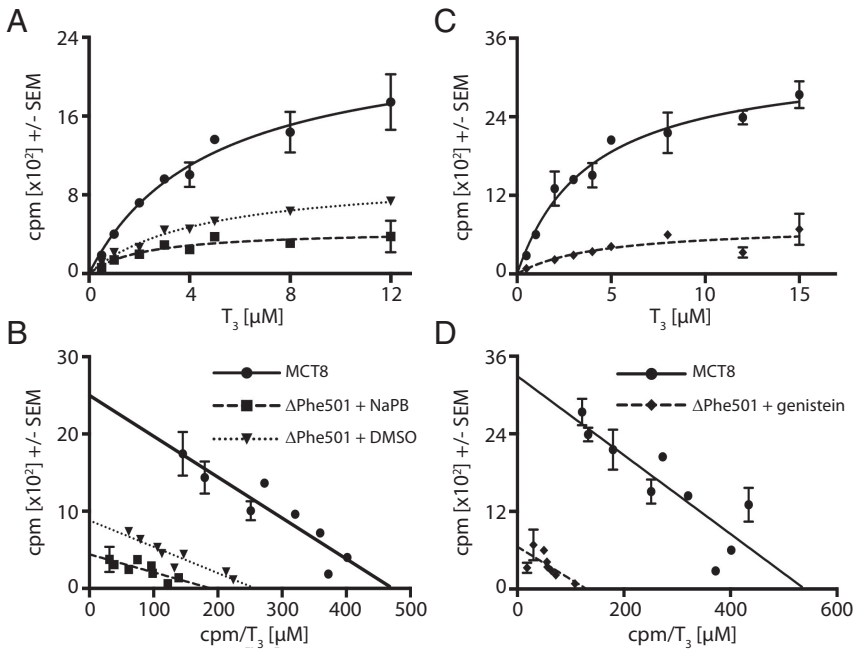

Figure 7. A) Determination of K_M values of treated $MCT8^{\Delta F501}$ compared to untreated wildtype MCT8. Treatment occurred with 4 mM NaPB and 2% DMSO, respectively, for two days. Empty vector-transfected cells were used as background controls and subtracted from saturation curves. Measurements were performed with increasing concentrations of T_3 for 3 minutes. B) Eadie-Hofstee plot of A with K_M(WT)= 4.8 $\pm$ 0.9 μM, K_M(ΔPhe501,DMSO)= 4.4 $\pm$ 0.6 μM, and K_M(ΔPhe501,NaPB)= 2.2 $\pm$ 0.8 μM C) Determination of K_M value of genistein (15 μM for two days) treated $MCT8^{\Delta F501}$. D) Eadie-Hofstee plot of C with K_M(WT) = 3.9 $\pm$ 0.6 μM and K_M(ΔPhe501, genistein) = 4.3 $\pm$ 2.4 μM. Measurements were performed with increasing concentrations of T_3 for 10 minutes.

Acknowledgments

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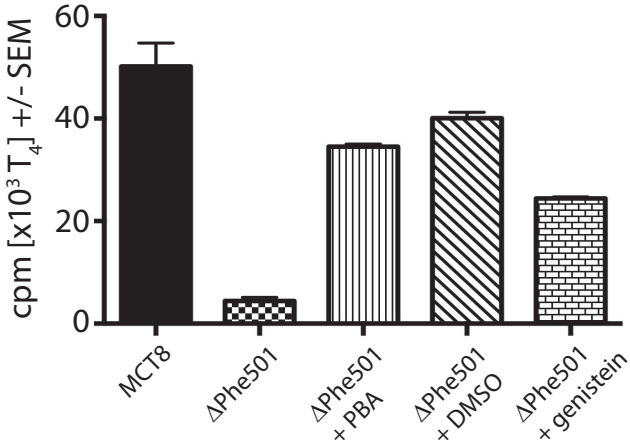

Figure 8. Chaperone treatment for two days also improves T_4 uptake. 10 nM ^{125}I- T_4 uptake in the presence of 4 mM PBA, 2% DMSO and 15 μM genistein. Wildtype MCT8 and empty vector-transfected cells served as positive and negative controls, respectively.

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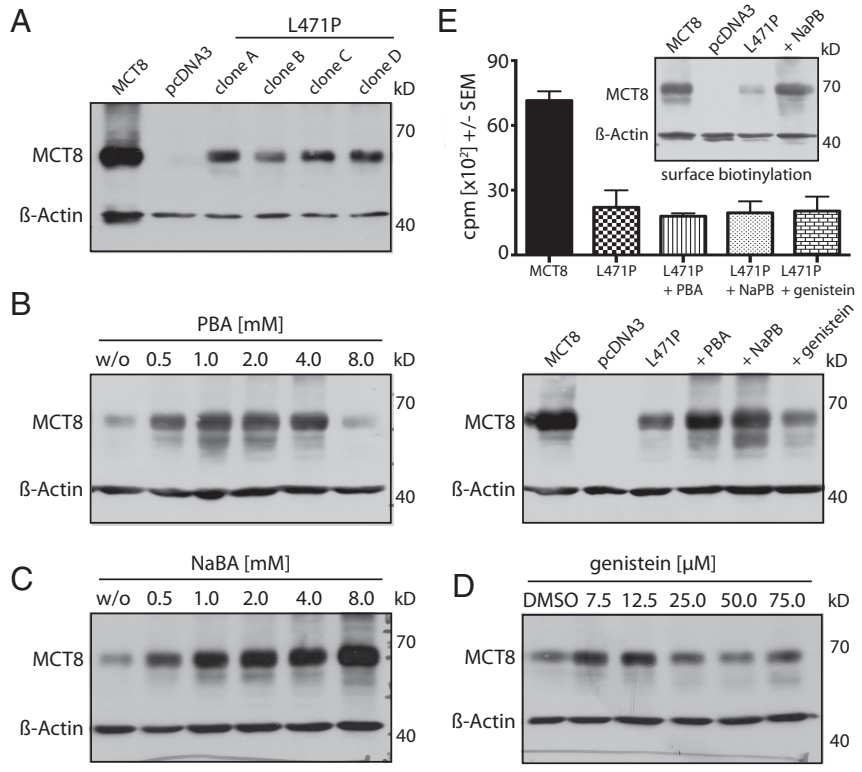

Figure 9. Treatment of the pathogenic mutation MCT8^{L471P} with chemical and pharmacological chaperones for two days. MCT8^{L471P} is associated with a severe phenotype in AHDS patients. A) Stable transfection of MDCK1 cells with MCT8^{L471P} led to several cell clones, which show a slight decrease in mutant MCT8 expression compared to wildtype. B+C) The treatment of MDCK1 cells stably expressing MCT8^{L471P} with 4PBA (B) and NaPB (C) for two days increased MCT8 expression in whole cell homogenates dose-dependently. ß-actin served as loading control. D) Genistein treatment for two days has no effect on mutant MCT8 expression. Western blot of total protein, ß-actin served as loading control. E) Lower panel: Expression of MCT8^{L471P} in MDCK1 cells is increased by 4 mM 4PBA and 4 mM NaBP but not by 15 μM genistein compared to MCT8^{WT}. Empty vector-transfected cells served as negative control. Western blot of total protein, ß-actin served as loading control. Upper panel: The treatment of MCT8^{L471P} with 4 mM 4PBA, 4 mM NaPB and 15 μM genistein for two days did not increase the uptake of 2 nM ^{125}I-T_3 although the mutant is expressed at the cell surface (surface biotinylation). Endpoint assay: t = 20 minutes.

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