Identification of chemical and pharmacological chaperones for correction of trafficking-deficient mutant CNGA3 channels

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ABSTRACT

Trafficking deficiency caused by missense mutations is a well-known phenomenon occurring for mutant, misfolded proteins. Typically, the misfolded protein is retained by the protein quality control system and degraded by the endoplasmic reticulum-associated protein degradation pathway and thus does not reach its destination although residual function of the protein may be preserved. Chemical and pharmacological chaperones can improve the targeting of trafficking-deficient proteins and thus may be promising candidates for therapeutic applications. Here, we report the application of a cellular bioassay based on the bioluminescent calcium reporter aequorin to quantify surface expression of mutant CNGA3 channels associated with the autosomal-recessively inherited retinal disease achromatopsia. A screening of 77 compounds enabled the identification of effective chemical and pharmacological chaperones resulting in a 1.5 to 4.8 fold increase of surface expression of mutant CNGA3. We confirmed that the rescue of the defective trafficking is not limited to a single mutation in CNGA3 using selected compounds. Active compounds and our structure-activity correlated data for the dihydropyridine compound class may provide valuable information for developing a treatment of the trafficking defect in achromatopsia.
Significance statement: We describe a novel luminescence-based assay to detect the surface expression of mutant, trafficking deficient CNGA3 channels based on the calcium sensitive photoprotein aequorin. Using this assay for a compound screening, we identified novel chemical and pharmacological chaperones restoring the surface localization of mutant trafficking-deficient CNGA3 channels. The results from our work may serve as starting point for the development of potent compounds rescuing trafficking deficiencies in the autosomal-recessively inherited retinal disease achromatopsia.
INTRODUCTION

Achromatopsia (ACHM) is a rare retinal disorder characterized by loss of cone photoreceptor function (Remmer et al., 2015). Six genes are associated with achromatopsia (Kohl et al., 1998; Kohl et al., 2000; Kohl et al., 2002; Chang et al., 2009; Kohl et al., 2012 and Kohl et al., 2015): CNGA3 (OMIM: 216900), CNGB3 (OMIM: 262300), PDE6C (OMIM: 613093), PDE6H (OMIM: 610024), GNAT2 (OMIM: 613856) and ATF6 (OMIM: 616517). Most patients carry mutations in CNGA3 and CNGB3 encoding for both subunits of the cyclic nucleotide-gated (CNG) channel expressed in the cone photoreceptor outer segment (Johnson et al., 2004). The cone CNG channel is a non-selective cation channel with a proposed stoichiometry of three CNGA3 and one CNGB3 subunit (Shuart et al., 2011). Both channel subunits are structurally homologous proteins consisting of six transmembrane helices (S1-S6), a pore region between S5 and S6, a ligand-binding domain for cyclic nucleotides (CNBD) and a C-linker connecting S6 with the CNBD. The main subunit is CNGA3 which can form functional homomeric channels in heterologous expression systems whereas CNGB3 is the modulatory subunit (Kaupp and Seifert, 2002).

In earlier studies, we and others performed functional analysis of mutant homomeric and heteromeric cone CNG channels in heterologous expression systems (Failace et al., 2004; Tränkner et al., 2004; Liu et al., 2005; Nishiguchi et al., 2005; Patel et al., 2005; Muraki-Oda et al., 2007; Koeppen et al., 2008; Reuter et al., 2008; Ding et al., 2010; Koeppen et al., 2010; Matveev et al., 2010; Duricka et al., 2012; Shaikh et al., 2015). Besides altered biophysical characteristics, pathogenic mutations were frequently shown to result in a reduced surface expression caused by a trafficking defect (Failace et al., 2005; Tränkner et al., 2004; Liu et al., 2005; Nishiguchi et al., 2005; Koeppen et al., 2008; Duricka et al., 2012; Shaikh et al., 2015). On the molecular level, the trafficking defect results from a protein folding deficit which is induced by the mutation (Conn and Janovick, 2009). During protein maturation the misfolded protein is recognized and retained by the cellular protein quality control system (Adams et al., 2019) and subsequently degraded. Trafficking defects have been identified frequently as pathological mechanism causing diseases (Tamarappoo and Verkman, 1998; Skach, 2000; Blom et al., 2003; Yam et al., 2005; Sato et al., 2009; Schaeffer et al., 2014; Kuech et al., 2016). However, in many cases the mutant protein is still functioning and could execute its cellular function.
when reaching its intended subcellular location (Gelsthorpe et al., 2008). Thus, developing a strategy to avoid the mutant protein being trapped and degraded may result in a rescue of the disease phenotype.

Chemical chaperones reverse defective trafficking by either nonspecifically changing protein-solvent interactions thus favoring correct folding (Leandro et al., 2008, Dandage et al., 2015) or increasing expression levels of heat shock proteins supporting protein folding (Diamant et al., 2001). An elevated surface density of trafficking-deficient mutant CNGA3 has already been observed in cell culture systems after treatment with chemical chaperones (Koeppen et al., 2008; Duricka et al., 2012). However, the use of chemical chaperones for therapeutic applications is restricted due to their nonspecific effect on protein folding and the high concentrations needed.

Pharmacological chaperones are typically agonists or antagonists of the mutant protein and stabilize the target protein in its native conformation by direct binding (Leidenheimer and Ryder, 2014; Wang et al., 2014). Due to their specific interaction, effective concentrations lie in the low micromolar or nanomolar range giving pharmacological chaperones a high potential for therapeutic purposes. The effectiveness of pharmacological chaperones is gaining traction, with a number of successful demonstrations of its use in recent years (Rigat and Mahuran, 2009; Dawson et al., 2010; Martin et al., 2013; Wang et al., 2014; Banning et al., 2016; Hoshina et al., 2020; Li et al., 2020). For trafficking-deficient cone CNG-channels with mutations in the CNBD domain it has been shown that treatment with an analogue of the natural ligand cGMP results in an increased surface expression (Duricka et al., 2012).

In the present study, we identified substances that support proper folding and trafficking of mutant CNGA3 channels. An aequorin-based bioassay was established enabling a relative quantification of the CNGA3 channel density in the plasma membrane via CNGA3-mediated calcium influx. This assay was used to screen nine known chemical chaperones and 68 compounds—among them a substantial number of dihydropyridine (DHP) compounds. Additional 12 DHP derivatives were assayed to provide insights into structural requirements of DHP compounds for correction of the trafficking defect of mutant CNGA3 channels.
METHODS

Plasmids

The generation of the wild type and mutant human CNGA3 (NM_001298.2) and CNGB3 (NM_019098.4) expression constructs were described previously (Tränkner et al., 2004; Koeppen et al., 2008; Reuter et al., 2008; Koeppen et al., 2010). The plasmid pCAEQ encoding for a mitochondrial localized apoaequorin was kindly provided by Perkin Elmer (Waltham, MA) and was modified by excision of the mitochondrial targeting sequence. Cytoplasmic localization was confirmed by co-localization of the myc-tagged apoaequorin with the mitochondrial markers Apoptosis-inducing factor (RRID:AB_726995) and Mitotracker Orange CMTMRos (Fig. S1).

Cell culture and transfection

The human embryonic kidney 293 (HEK293, RRID:CVCL_0045) cells were maintained in high glucose Dulbecco's Modified Eagle Medium (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Life Technologies Corporation, Carlsbad, CA, USA), 1% fungizone (PAA, Linz, Austria) and 1% penicillin/streptomycin (Sigma Aldrich GmbH, Munich, Germany) at 37°C and 5% CO₂. HEK293 cells were seeded at a density of approximately 2 x 10⁵ cells/cm² and transfection was carried out using lipofectamine 2000 (Life Technologies Corporation, Carlsbad, CA, USA). For bioassay and cell viability experiments, plasmids encoding for the wild type CNGA3 channels or CNGA3_E228K channels and apoaequorin were co-transfected in a ratio of 1.0 : 2.1. For all remaining mutant CNG channels, a ratio of 1.0 : 0.3 : 2.1 for CNGA3, CNGB3 and apoaequorin was chosen. For Western blot experiments, HEK293 cells, at a confluence of 80%, were transfected with 7.5 µg plasmid DNA in a 6-well.

Six hours post-transfection, HEK293 cells were washed with Dulbecco's phosphate buffered saline (DPBS; Life Technologies Corporation, Carlsbad, CA, USA), detached by treatment with Trypsin-EDTA (Life Technologies Corporation, Carlsbad, CA, USA) and seeded in opaque 96-well plates at a density of 7.5 x 10⁴ cells per well for the aequorin-based bioassay and the cell viability experiments or 6 cm dishes for Western blot experiments. 24 h post transfection, cells were treated with 3 mM sodium butyrate (Sigma Aldrich GmbH, Munich, Germany) and the test compound over a duration of 24 h.
For any experiment, cells used for negative control, positive control and treatment with a certain compound originated from the same transfection.

**Aequorin-based bioassay**

Cells were washed with DPBS and incubated with 8 µM coelenterazine (Biomol GmbH, Hamburg, Germany) in calcium imaging solution (5 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 10 mM HEPES, 30 mM Glucose, 150 mM NaCl, pH 7.4) for 4 h to reconstitute the aequorin. Cells were washed with DPBS and 90 µl of calcium imaging solution containing 10 mM CaCl$_2$ was added. Luminescence measurements were carried out using an Orion luminometer (Titertek-Berthold Detection Systems, Pforzheim, Germany) having a spectral range of 300 – 650nm. Luminescence was recorded for 50 s and 10 µl of 100 mM 8-bromoguanosine-3',5'-cyclic monophosphate (8-Br-cGMP; BIOLOG Life Science Institute, Bremen, Germany) was applied 3 s after the measurement started. For data evaluation, the base line luminescence before addition of the ligand 8-Br-cGMP (basal calcium flux) was subtracted for each measurement. The area under the curve (AUC) was calculated for an interval of 26 s starting 8 s after application of the cGMP analogue for cells expressing CNGA3$_{WT}$ or CNGA3$_{E228K}$ channels and a 26 s interval starting 16 s after ligand application for cells expressing other CNGA3 mutants. By this means, we only included the values corresponding to the peak of the calcium transient for analysis excluding any application artifacts. For analyzing the data obtained by the compound screen, the fold change (FC) of the calculated AUC luminescence responses of the treated versus untreated cells or of wild type versus mutant channels was determined.

**Screening and experiments with wild type and additional mutant CNGA3 channels**

To evaluate the effect of chaperone treatment, solvent-treated cells (designated as untreated control throughout the manuscript) were used as control. Treatment with 2.5 % glycerol served as positive control for cells expressing wild type or mutant CNG channels. Concentrations of chemical chaperones ranged between 0.5 to 850 mM. Ion channel antagonists and their derivatives were first screened in quadruplicates using 3.3 µM, 10 µM, 33 µM and 100 µM of the compounds. Compounds showing a FC of about 1.0 at all tested concentrations were re-tested at higher concentrations whereas compounds showing a FC smaller than 1.0 at all concentrations indicating toxicity of the compound were re-tested at lower concentrations. In order to classify compounds as active, we defined an
arbitrary fold change (FC) cut-off value of 1.5. Activity of compounds was confirmed in two independent experiments using six concentrations close to the estimated signal maximum. The follow-up experiments using wild type and additional trafficking-deficient CNGA3 were done in quadruplicates and in two independent experiments.

**Cell viability assay**

Compounds were tested in their most effective concentration determined by the bioassay. After compound treatment, cells were washed with DPBS and 50 µl of calcium imaging solution was added to the wells. Subsequently, the CellTiter-Glo® Luminescent Cell Viability Assay (Promega GmbH, Mannheim, Germany) was performed following the manufacturer’s instructions using the Orion luminometer. Two independently transfected cell populations were tested in quadruplicates and statistical analysis was performed as described below.

**SDS-PAGE and Western blot experiments**

Cells were trypsinized, washed gently with DPBS buffer and transferred into ice cold lysis buffer (1 mM DTT, 1 mM EDTA, 50 mM Tris and 150 mM NaCl, pH 7.4) containing 1 % proteinase inhibitor cocktail (Merck Millipore, Billerica, MA). Cell lysis was performed by four freeze / thaw cycles using liquid nitrogen. After cell debris removal by centrifugation at 500 x g for 10 min at 4°C, membrane fractions were enriched by centrifugation at 30 000 x g for 45 min at 4°C. Pellets were resuspended in 50 µl lysis buffer containing 1 % NP-40 and 0.25 % sodium deoxycholate and the protein concentration was determined using Bradford assay. Total protein lysates were incubated with Laemmli buffer (60 mM Tris HCl, 10 % glycerol, 5 % 2-mercaptoethanol, 2 % SDS, 0.01 % bromphenol blue) for 1 h at 4°C. Samples were separated on a 10 % SDS-polyacrylamide gel. After transfer onto nitrocellulose membranes, blots were blocked overnight with 5 % milk powder (Bio-Rad Laboratories GmbH, Munich, Germany) in Tris-buffered saline supplemented with 0.1 % Tween® 20 and probed with a mouse anti-c-myc antibody (1:700; RRID:AB_10541551; Enzo Life Sciences, Lörrach, Germany) for the detection of apoaequorin, the custom-made rabbit-anti-CNGA3 antibody SA3899 or mouse anti-β-actin antibody (1:4000; RRID:AB_2223041; Merck Millipore, Billerica, MA) as loading control. For detection, a HRP-conjugated goat-anti-mouse (1:10 000; RRID:AB_437779; Merck Millipore, Billerica, MA) as well as a HRP-conjugated donkey-anti-rabbit (1:4000;
RRID:AB_772206; GE Healthcare Life Sciences, Freiburg, Germany) secondary antibody were used.

Protein bands were quantified using ImageJ (RRID:SCR_001935; National Institutes of Health, Bethesda, MD, USA).

**Statistical data analysis**

For statistical analysis of the bioassay results, the FC of treated samples versus untreated controls was calculated. For bioassay experiments and the quantification of the Western blots, fold changes are depicted as mean and 95% confidence interval (mean ± CI) throughout the whole document. For the cell viability assay, the viability of the untreated cells was set to 100% and treated samples referred to that. Box plots are shown as median and upper as well as lower quartile. The whiskers represent the maximum and minimum value for each data set. Statistical analysis was performed using the Mann-Whitney U test for pairwise comparison of each compound treatment with the untreated control using Mystat (Systat Software Inc., London, UK) and the Kruskal-Wallis one-way analysis of variance (ANOVA) with the Dunns Post-hoc test was applied for multiple comparison (GraphPad Prism, San Diego, USA). Statistical significance of the data was defined as follows: *: p<0.05; **: p<0.01; and ***: p<0.001. This study is exploratory by nature.
RESULTS

Identification of compounds increasing the luminescence signal in the bioassay and effect of six most potent compounds on cell viability

Homomeric CNGA3 channels are integrated into the plasma membrane of transfected HEK293 cells and conduct ions along their concentration gradient after addition of the CNG channel ligand 8-Br-cGMP. This calcium flux can be monitored using the genetically encoded calcium-sensitive photoprotein aequorin residing in the cytoplasm. The ligand-induced calcium flux results in a strong increase of the luminescence which is absent in cells not expressing CNG channels and thus solely represents CNG channel mediated calcium flux as shown previously (Täger et al., 2018) and in Fig. S2. Using this luminescence-based bioassay we performed a screening of nine known chemical chaperones and 65 ion channel antagonists and three DHP derivatives for their potential to increase the luminescence signal compared to the untreated control. The ion channel antagonists were selected manually based on known interaction with CNGA3 or other cation channels and their commercial availability.

For the screening, CNGA3 channels with the mutation p.E228K which is located in the intracellular loop connecting S2 and S3 were used. The mutation p.E228K is primarily impaired by a trafficking defect since the apparent ligand sensitivity was found to be similar to wild type channels (Reuter et al., 2008). Also, the impact of the mutation p.E228K onto CNG channel functionality is rather mild, since CNGA3\textsubscript{E228K} channels conduct ions as observed by electrophysiology (Reuter et al., 2008) and the bioassay described here (Täger et al., 2018). Due to this residual activity, toxic side effects of compounds were detected. As positive control throughout the screening we used glycerol which has already been shown to possess a beneficial effect on surface expression of mutant CNGA3 channels (Koeppen et al., 2008). A table showing all compounds and the results of the bioassay is available in the supporting information (Table S1).

In order to only classify medium and strong compound effects as active, we considered compounds reaching a FC of 1.5 or higher as active. The screening was performed using initially four concentrations of the test compounds, if compound activity was observed the compound was re-tested twice with six concentrations. If the compound treatment caused toxicity, lower concentrations were
tested and if the FC of all tested concentration did not deviate from 1.0, higher concentrations were used. Typically, only the FC of the compound concentration yielding the highest effect is presented (FC$_{\text{max}}$).

Of the nine chemical chaperones tested, only after treatment with 100 mM L-glutamine a compound activity with a FC$_{\text{max}}$ of 1.5 ± 0.2 was observed (Fig 1A). The other chemical chaperones did not show activity. Based on their molecular structure, the ion channel antagonists tested were subdivided in several classes. Of those, the largest class consisting of 17 compounds was the dihydropyridine (DHP) class inhibiting preferentially L-type calcium channels. Eight of the DHPs (47 %) showed activity with a FC$_{\text{max}}$ ≥ 1.5, of those nisoldipine having the highest effect with a FC$_{\text{max}}$ of 4.8 ± 0.7 at 20 µM (Fig. 1A). The effect of 15 µM JFD03311 having a FC$_{\text{max}}$ of 2.4 ± 0.1 is considerably lower compared to nisoldipine, the other six active DHPs had FC$_{\text{max}}$ between 1.5 and 2.0. The lowest concentration necessary to reach FC$_{\text{max}}$ was obtained after treatment with 0.66 µM niguldipine (FC$_{\text{max}}$ of 1.6 ± 0.1).

Another class of pharmacological chaperones comprised five pyrazin amides and benzamides which showed to be active having FC$_{\text{max}}$ between 1.5 ± 0.1 (10 µM cisapride) and 3.8 ± 0.6 (400 µM amiloride). Of the remaining 46 non-DHPs, 11 compounds (24 %) showed activity. Among them were the N-phenylsulfonamide zatebradine, a hyperpolarization-activated cyclic nucleotide-gated (HCN) channel antagonist, with a FC$_{\text{max}}$ of 2.5 ± 0.2 at 400 µM and the human ether-a-go-go-related gene (hERG) potassium channel antagonist E-4031, a high molecular-weight compound containing dimethoxybenzene groups, with a FC$_{\text{max}}$ of 1.7 ± 0.2 also at 400 µM. Additionally, the HCN channel antagonist ZD7288 and the voltage-gated sodium channel antagonist bupivacaine were identified as active hits, ZD7288 with a FC$_{\text{max}}$ of 2.2 ± 0.1 at 200 µM and bupivacaine with a FC$_{\text{max}}$ of 2.0 ± 0.2 at 400 µM.

The dose-dependent effects of the six compounds with highest FC$_{\text{max}}$ (nisoldipine, JFD03311, amiloride, zatebradine, ZD7288 and bupivacaine with FC$_{\text{max}}$ ≥ 2.0) are plotted in Fig. 1B. With the exception of nisoldipine, we observed a moderate increase in FC with increasing compound concentration until reaching FC$_{\text{max}}$. In case of amiloride, bupivacaine and zatebradine, the concentration eliciting the maximum FC was the highest concentration tested while for JFD03311 and
ZD7288 the luminescence signal decreased after reaching FC\textsubscript{max}. In contrast, nisoldipine showed a rapid increase and decline in FC in a concentration range which is much smaller than for the other compounds. It is also striking that the concentrations needed to reach FC\textsubscript{max} are lower for the DHPs nisoldipine and JFD03311 compared to the non-DHPs.

The six compounds with highest FC\textsubscript{max} were further evaluated by testing for toxic side effects at their most effective concentration using a luminescence-based cell viability assay. Both, JFD03311 and amiloride did not affect cell viability, whereas treatment with nisoldipine, zatebradine or bupivacaine caused a reduction of cell viability by 10 – 20 % with p < 0.001. However, the strongest effect was observed after treatment with ZD7288 which reduced cell viability by more than 30 % (Fig. 2).

**Effect of pharmacological chaperones onto the expression of CNGA3\textsubscript{E228K} and apoaequorin**

We performed follow-up experiments to confirm that the observed increase in the luminescence signal following compound treatment indeed is a result of improved CNG channel trafficking. For that purpose, cells treated with the six compounds at the concentration yielding the highest FC\textsubscript{max} were subjected to Western blot experiments to analyze the expression levels of CNGA3\textsubscript{E228K} and the reporter apoaequorin. We observed that treatment of cells with glycerol, zatebradine and ZD7288 caused a 2-fold increase in the expression level of apoaequorin, JFD03311 provoked a 5-fold increase (p<0.05) and amiloride, bupivacaine and nisoldipine induced a 10- to 25-fold increase (p<0.05) in apoaequorin expression (Fig. 3A). Expression levels of CNGA3\textsubscript{E228K} were also increased after treatment with all compounds, however the effects were milder compared to apoaequorin with a 1.5- to 2.0-fold increase of CNGA3\textsubscript{E228K} (Fig. 3B). Thus, the high FC\textsubscript{max} observed for some compounds in the bioassay using CNGA3\textsubscript{E228K} may have been caused, at least partially, by affecting the expression levels of CNGA3\textsubscript{E228K} and apoaequorin.

In order to evaluate the effect of the overexpression on the bioassay signals, we used cells expressing the wild type CNGA3 channel which is not impaired by a trafficking defect. Any increase in the luminescence therefore would reflect the effect of CNGA3 and apoaequorin overexpression. For this experiment we focused on nisoldipine, amiloride, bupivacaine and JFD03311 which all showed at least a 5-fold increase in apoaequorin levels. Nisoldipine (FC = 2.7 ± 0.1), amiloride (FC = 2.1 ± 0.2)
and bupivacaine (FC = 1.9 ± 0.1) treatment indeed caused a strong increase of the luminescence signal after treatment of CNGA3<sub>WT</sub> correlating with the higher apoaequorin expression observed in the Western blot experiments (Fig. 4). This increase was not present after treatment with JFD03311. In order to verify that – despite the overexpression effects – a rescue of CNGA3<sub>E228K</sub> channel trafficking is conferred by compound treatment, we compared the fold changes obtained with CNGA3<sub>E228K</sub> and CNGA3<sub>WT</sub>. Luminescence signals following treatment of CNGA3<sub>E228K</sub> with nisoldipine, amiloride or JFD03311 were at least > 1.7-fold higher (p<0.001) compared to treatment of CNGA3<sub>WT</sub>. No difference was observed following treatment of CNGA3<sub>WT</sub> or CNGA3<sub>E228K</sub> with bupivacaine (Fig. 4). Thus, we confirmed that the overexpression effect is not the major cause for hit identification of DHP compounds in the luminescence-based assay. Furthermore, we showed that for five additional compounds no or only a minor overexpression effect was observed (Fig. S3).

**Rescue of other trafficking-deficient CNGA3 channels**

Typically, pharmacological chaperones are able to correct the folding and trafficking defect caused by different mutations in the protein of interest (Wu et al., 2011; Wang et al., 2014). In order to test this hypothesis, we treated cells expressing trafficking-deficient CNGA3 channels carrying missense mutations in S4 (p.R283Q, p.T291R), the pore forming domain (p.S341P, p.E376K), the linker (p.R427C) and the ligand-binding domain (p.R563C) with amiloride, JFD03311, nisoldipine, zatebradine and ZD7288 at their most effective concentrations identified in the initial screening using CNGA3<sub>E228K</sub> (Table1). For simplification, we only show the corrected fold change for each treatment based on the experiment using wild type CNGA3.

Amiloride was able to rescue the trafficking defect of all mutant CNGA3 channels while the rescue effect of ZD7288 and zatebradine was limited to mutations located in S4 and the pore forming region. Treatment with the DHP nisoldipine was able to correct the misfolding of CNGA3<sub>T291R</sub> and CNGA3<sub>S341P</sub> but also resulted in low or even negative FC for three out of four of the remaining CNGA3 channel mutants tested. JFD03311 corrected the misfolding of both S4 channel mutants, CNGA3<sub>S341P</sub> and CNGA3<sub>R427C</sub>. The highest FC were observed for amiloride and ZD7288 when expressing CNGA3<sub>S341P</sub> channels with values of 5.0 ± 0.4 and 3.7 ± 0.3, respectively.

**Analysis of structural features improving DHP activity**
Since many compounds analyzed in this study belong to the dihydropyridine class of compounds and additional derivatives were commercially available, we aimed to investigate the structure-activity relationship of the DHP. For that purpose, 12 additional DHPs with distinct modifications at R_1 to R_3 of the dihydropyridine backbone were included (Fig. 5A). A summary of the FC_{max} obtained for all 28 tested DHPs is shown in table S2. The structural features of these DHP derivatives are summarized in Table S3.

In Fig. 5B we give an overview of all compound classes tested which includes compounds with modification on the benzene ring, the dihydropyridine ring and at the dihydropyridine nitrogen on FC_{max}. The modifications on the benzene ring were subdivided in three categories: Many of the DHPs harbor a nitro group at the benzene ring which may serve as an electron acceptor for the interaction with mutant CNGA3 channels. Others carry an unmodified or a halogen-substituted benzene ring or, alternatively, a large polar residue at the benzene ring.

In order to understand which of the compound modification is important for the interaction with CNGA3_{E228K}, a comparison of the FC_{max} of the DHP groups was performed. With the exception of the DHPs sharing an unmodified or a halogen-substituted benzene ring (average FC_{max} = 1.2), all other compound classes yielded an FC_{max} of 1.5 or higher. The highest effects yielded DHP with a nitro group at the benzene ring with an average FC_{max} of 2.0 in the bioassay. We performed an ANOVA analysis and could show that the analyzed compound groups possess statistically significant differences among groups (p = 0.006). DHPs having a nitro group at the benzene ring exhibited a 1.7-fold higher FC_{max} (p < 0.05) compared to unmodified or halogen-substituted DHPs indicating that a nitro group at R_1 is beneficial for their interaction with CNGA3_{E228K}. Adding a larger substituent at the dihydropyridine nitrogen at R_3 also increased FC_{max} by a factor of 1.6 (p<0.05) compared to the unmodified or halogen-substituted DHPs. Modifications at the dihydropyridine ring such as altered ester side chain modifications or the presence of a oxocyclohexene ring had only minor effects on FC_{max}. In summary, addition of a nitro group at the benzene ring or adding residues at the dihydropyridine nitrogen represents an efficient strategy to increase the effect of compounds on mutant CNGA3_{E228K} channels.
DISCUSSION

This study aimed to identify chemical and pharmacological chaperones correcting the trafficking defect of mutant CNGA3 channels. In order to establish a robust assay for screening, we used a functional readout based on the CNGA3 channel mediated calcium influx. This enabled the indirect quantification of CNGA3 channels localized in the plasma membrane. HEK293 cells do not express endogenous CNG channels or other cyclic nucleotide activated calcium channels. Thus, the strong calcium signal elucidated by the application of 8-Br-cGMP in our bioassay solely represented the function of the transiently expressed CNGA3 channels (Fig. S2). For relative calcium quantification, the genetically encoded calcium sensor aequorin was used allowing the detection of calcium exclusively in the cell cytoplasm thus minimizing background signal (Brini, 2008). The bioluminescent aequorin has been used previously for assay development and screening (Menon et al., 2008; Haq et al., 2013; Arduino et al., 2017).

For compound screening, the FC of the calcium signal obtained in the treated condition versus the calcium signal of an untreated control was calculated. We defined compounds as active when yielding a p-value of < 0.05 and a FC cut-off value of ≥ 1.5 fold. This allowed the identification of strong hits and the removal of hits with minor effects (Fig.1). The screening of 77 compounds resulted in the identification of 22 chemical and pharmacological chaperones (hit rate: 29 %). Compared to other screening campaigns, this hit rate is extremely high (Nühs et al., 2015; Atzmon et al., 2018; Stevens et al., 2019) but can be explained by: (i) We specifically selected known ion channel antagonists for the screening. (ii) A large number of active compounds were structurally related. (iii) We tested each compound in a broad concentration range with 4 to 6 compound concentrations.

By testing nine selected chemical chaperones, we identified L-glutamine as hit. The activity of L-glutamine may rely on its ability to enhance the expression of the stress-inducible heat shock protein 70 (Moura et al., 2018) and increased amounts of this protein have already been shown to reduce protein misfolding (Choo-Kang and Zeitlin, 2001; Young, 2014). Another chemical chaperone tested was TUDCA which has been reported to increase surface localization of heteromeric CNG channels with the mutations p.R563C and p.Q655X in CNGA3 (Duricka et al., 2012). Although we obtained a statistically significant increase in the FC of 1.3 after treatment with 3.3 mM TUDCA indicating an
increased surface localization of CNGA3<sub>E228K</sub>, TUDCA was classified as inactive due to our stringent hit selection criteria.

For many screening campaigns, the hits identified in primary screening projects cannot be replicated. This may be due to false positive hits caused by the experimental settings or compound properties (Gilberg et al., 2016; Horvath et al., 2016; Jasial et al., 2017). In this regards, we can exclude that the compounds studied here had effects on the biophysical properties of the CNGA3 channel since compounds were washed out before performing the functional assay. By subtraction of the baseline signal from each measurement before analyzing the calcium signal, we can rule out any effects of changes in the basal calcium levels on our analysis. Additionally, we investigated the validity of the results obtained for the six best hits by analyzing whether these compounds altered the expression of CNGA3<sub>E228K</sub> or apoaequorin (Fig. 3). Indeed, we observed for amiloride, bupivacaine, JFD03311 and nisoldipine an increased abundance of apoaequorin in Western blot experiments. This might be a result of elevated transcription / translation, increased protein stability or interference with degradation of apoaequorin. Interestingly, the effect onto CNGA3<sub>E228K</sub> abundance was milder: a 1.3- to 2-fold increase was observed for all compounds, however statistically significant effects were only present after treatment with amiloride and JFD03311.

To study how this effect would affect the luminescence signal, we repeated the compound treatment and bioassay using wild type CNGA3 channels that do not have a trafficking defect. We saw an increase in fold change after treatment with amiloride, JFD03311, nisoldipine and bupivacaine. However, for amiloride, JFD03311 and nisoldipine the FC obtained using CNGA3<sub>E228K</sub> channels was > 1.5-fold higher than that of wild type CNGA3 expressing cells suggesting that beside the overexpression effect these compounds also improved surface trafficking of the mutant channel. This was not true for bupivacaine indicating that this compound represents a false positive hit.

Using additional mutant CNGA3 channels, we confirmed that the identified compounds can also correct the trafficking defect induced by the mutations p.R283Q, p.T291R, p.S341P, p.E376K, p.R427C and p.R563C (Table 1). Since CNGA3<sub>S341P</sub> and CNGA3<sub>E376K</sub> channels have previously been shown to be only functional in the presence of the CNGB3 subunit (Koeppen et al., 2010), we assayed all test compounds using cells expressing heteromeric CNG channels. For most of the five compounds,
an improvement of the trafficking of CNGA3 channels with mutations in the transmembrane helices or the pore-forming region was observed. This is not surprising since many of the tested antagonists interact with residues at the extracellular site or close to the center of the pore of the respective ion channels (Nakayama and Kuniyasu, 1996; Kamiya et al., 2008, Catterall and Swanson, 2015; Tanguay et al., 2019) and we propose that this is also the side of interaction with the CNGA3 channel for most of the tested compounds. Amiloride was shown to bind to the ENaC channel in a region close to the pore (Schild et al., 1997). Thus it is surprising that amiloride was able to rescue CNGA3<sub>R563C</sub> channels. Since this mutation is located in CNBD, we speculate that amiloride may have a second binding site close to this mutation. Overall, we confirmed that pharmacological chaperones were able to rescue several different mutant CNGA3 channels in the presence of the CNGB3 subunit – an observation which may also be important with respect to therapeutic applications.

Correcting the surface expression of trafficking-deficient ion channels is only possible when the compound enters the cell to bind to the mutant ion channel. Since many ion channel antagonists bind extracellularly to the ion channel, modification of compound structures to increase membrane permeability and specificity are inevitably. We undertook first experiments to elucidate the functional groups that are essential for interaction of DHP compounds with CNGA3<sub>E228K</sub> (Fig. 5). We could show that the nitrophenyl group is essential for efficiency of DHP and larger, polar substitutions at the DHP nitrogen yielded > 1.6-fold higher FC<sub>max</sub> compared to DHP with unmodified or halogen-substituted benzene rings. Similar to our study, the identification of structural features of DHP important for antagonizing N-type calcium channels (Yamamoto et al., 2008) or the potentiation of CFTR channels (Pedemonte et al., 2007) were reported. In both studies halogenated phenyl rings yielded better effects than nitrophenyl groups indicating that this feature is important for the specific interaction with CNGA3 channels.

ZD7288 yielded a FC<sub>max</sub> of 2.2 ± 0.1 at a concentration of 200 µM. However, we observed high toxicity in the cell viability assay (Fig. 2) resulting in a loss of about 30% of the cells which is also reflected in the bioassay experiments using wild type CNGA3. Therefore, the effect of ZD7288 seems to be specific and is underestimated due to its toxicity. Amiloride at 400 µM yielded the highest fold increase measured with the bioassay when using CNGA3<sub>S241P</sub> channels (FC of 5.0 ± 0.4). Despite its
dramatic impact on the expression of the reporter apoequorin, amiloride treatment did not induce toxic side effects and was able to correct the trafficking defect of all channel mutants tested. Interestingly, all amiloride derivatives were classified as active and those with higher molecular weight reached $\text{FC}_{\text{max}}$ at much lower concentrations (e.g. 2,4-dichlorbenzamil at 1 µM) (Fig. S4). Nisoldipine and JFD03311 caused both an increase in apoequorin expression in Western Blot experiments but only the effect of nisoldipine and not JFD03311 was shown to impact the bioassay measurements (Fig. 4). Also, the effect of nisoldipine on other trafficking-deficient channels was inconsistent while JFD03311 was able to increase surface expression of analyzed CNGA3 channels with mutations in the S4 region or the pore forming region. Therefore, we conclude that ZD7288, the pyrazin amides and benzamides and the DHP including JFD03311 may represent interesting starting points supporting the development of therapeutics for trafficking deficient CNGA3 channels.
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AUTHOR CONTRIBUTIONS

Participated in research design: Kohl, Reuter and Wissinger

Conducted experiments: Täger

Contributed new reagents or analytical tools: N/A

Performed data analysis: Reuter and Täger

Wrote or contributed to the writing of the manuscript: Kohl, Reuter, Täger and Wissinger
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FOOTNOTES

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FIGURE LEGENDS

Figure 1: Result of compound screening. A. HEK293 cells expressing the reconstituted calcium sensor aequorin and the mutant CNGA3_{E228K} channel were used for the screening. Treatment with nine known chemical chaperones, 17 dihydropyridines (DHP), five pyrazin amides / benzamides and 46 non-DHP was carried out in order to identify active chemical and pharmacological chaperones for mutant CNGA3 channels. The fold change represents the calcium signal obtained by treatment with most efficient compound concentration (FC_{max}) divided by the calcium signal obtained for the untreated control is shown. In order to be classified as “active”, compounds needed to show an FC_{max} of ≥ 1.5 and a p-value of < 0.05. Table S1 gives an overview of the results for each compound measured with this bioassay. B. Graphical representation of dose-response curves for the six identified best hits: amiloride, bupivacaine, JFD03311, nisoldipine, zatebradine and ZD7288 (n ≥ 4; *: p < 0.05; **: p < 0.01; ***: p < 0.001).

Figure 2: Analysis of cell viability after compound treatment. HEK293 cells were treated with amiloride, ZD7288, zatebradine, bupivacaine, nisoldipine and JFD03311 at their most effective concentration. Cell viability was determined by measuring total ATP content per well (n = 8; ***: p < 0.001).

Figure 3: Investigation of effects of compound treatment on apoaequorin and CNGA3_{E228K} expression levels. HEK293 cells expressing either A. apoaequorin or B. CNGA3_{E228K} were treated with the compounds that showed the highest effects in the primary screen at their most effective concentrations. Glycerol being the positive control in the screening procedure was included as well. Expression levels were quantified using membrane-enriched fractions of three replicates per treatment (*: p < 0.05, data represented as mean ± 95 % confidence interval).

Figure 4: Effect of compound treatment on the wild type CNGA3-mediated bioassay signal. CNGA3_{WT} is not affected by a trafficking defect. HEK293 expressing CNGA3_{WT} channel were used in order to measure increases in bioassay signal which are caused by unspecific effects due to compound treatment and the resulting fold changes compared to the values obtained by using CNGA3_{E228K} expressing cells. For nisoldipine and amiloride the fold change obtained by using the mutant CNGA3
channel is still statistically significant higher (n = 8; **: p < 0.01; ***: p < 0.001, data represented as mean ± 95% confidence interval). The data are summarized in the table below.

**Figure 5: Determining structural requirements of dihydropyridines (DHP) for improving CNGA3<sub>E228K</sub> interaction.** The effect of DHP with certain structural features was evaluated on HEK293 cells expressing aequorin and CNGA3<sub>E228K</sub>-A. Representation of common structural features of DHP compounds and location of modifications on residues R<sub>1</sub>-R<sub>3</sub> as described in B. B. Effects of DHP modifications on R<sub>1</sub>-R<sub>3</sub> on FC<sub>max</sub>. Statistical analysis showed that DHP with a nitrobenzene group and DHP with larger substitutions at the dihydropyridine nitrogen possess higher FC<sub>max</sub> than DHP with unmodified or halogen-substituted benzene rings. (n ≥ 4; Kruskal-Wallis one-way analysis of variance p=0.006 and Dunn’s Post-hoc test: *: p < 0.05).
Table 1: Effect of compound treatment on additional mutant CNGA3 channels. HEK293 cells expressing reconstituted aequorin and heteromeric trafficking deficient CNGA3 channels with mutations in transmembrane helix S4 (p.R283Q, p.T291R), the pore forming region (p.S341P, p.E376K), the linker (p.R427C) or the ligand binding domain (p.R563C) were treated with 400 µM amiloride, 200 µM ZD7288, 200 µM zatebradine, 20 µM nisoldipine or 15 µM JFD03311. Fold changes (FC) of treated versus untreated samples are shown (n = 8, data represented as mean ± 95% confidence interval). Results with FC ≥ 1.5 were considered as active (highlighted in grey).
Figure 1
Figure 2
Figure 3
Figure 4

Fold change

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. [μM]</th>
<th>FC for CNGA3 _E228K</th>
<th>FC for CNGA3 _WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisoldipine</td>
<td>20</td>
<td>4.8 ± 0.7</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Bupivacaine</td>
<td>15</td>
<td>4.0 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Amiloride</td>
<td>400</td>
<td>3.8 ± 0.6</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>

n.s. (no significant difference)

*** (p < 0.001)
Figure 5