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Functional rescue of vasopressin V2 receptor mutants in MDCK cells by pharmacochaperones: relevance to therapy of nephrogenic diabetes insipidus

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functional rescue of V2R mutants under such conditions. Moreover, as the V2R is expressed in the basolateral membrane (BM) of renal principal cells, and proteins can traffic or function differently in nonpolarized vs. polarized cells (17), such studies are best performed in polarized renal epithelial cells.

Recently, we generated Madin-Darby canine kidney (MDCK) cells stably expressing V2R tagged with a green fluorescent protein (GFP) (23). In these cells, V2R-GFP was localized and regulated as can be anticipated to occur for V2R in vivo. Moreover, we found that several V2R mutants in NDI stably expressed in MDCK cells are ER retained (24). To determine which CPAn is likely the optimal pharmacological chaperone to relieve NDI in patients, we thoroughly tested a two high-affinity V2R antagonists for their ability to rescue the cell surface expression and activity of several V2R mutants.

MATERIALS AND METHODS

Pharmacological chaperones. The V2R antagonist SR121463B (28) and the V1R antagonist SR49059 (29) were kindly supplied by C. Serraide-Legall (Sanofi Synthélabo, Toulouse, France). The V2R antagonists OPC31260 and OPC41061 (14, 35) were kindly provided by Koji Komuro (Otsuka Pharmaceutical, Tokushima, Japan). All compounds were dissolved in dimethylsulfoxide as 0.01 M stock solutions and diluted in culture medium as indicated.

Expression constructs, cell culture, and transfection. Expression constructs encoding the wild-type V2R or the NDI-causing mutants -L44P, -I130F, -S167T, or -S167L fused at their COOH terminus to enhanced GFP were as described elsewhere (24). MDCK type II cells, which lack endogenous V2R expression, were kindly provided by Dr. Alexander Oksche (FMP, Berlin, Germany). MDCK type II cells were maintained in DMEM (Biowittaker, Verviers, Belgium) supplemented with 5% fetal bovine serum (PAA Laboratories, Karlsruhe, Germany), gentamicin, 1-glutamine, sodium carbonate, and 1% nonessential amino acids. Calcium phosphate transfection and isolation of clones were done as described for MDCK type I cells (9).

Immunoblotting and immunocytochemistry. Polycrylamide gel electrophoresis, Western blotting, and immunodetection were performed as described elsewhere (9, 23). For detection of V2R-GFP, 1.5,000 diluted rabbit anti-GFP serum was used (kindly provided by Prof. B. Wieringa, RUN-MC, Nijmegen, The Netherlands). As secondary antibodies, horseradish peroxidase-coupled goat anti-rabbit IgGs (Sigma) were used. Immunocytochemistry, confocal laser-scanning microscopy (CLSM), and data quantification were performed as described elsewhere (24). As primary antibodies, 1:100-diluted rat anti-E-cadherin (Sigma, St. Louis, MO) or rabbit anti-protein disulfide isomerase antibodies (kindly provided by Dr. I. Braakman, Utrecht University, Utrecht, The Netherlands) were used. As secondary antibodies, 1:100-diluted goat anti-rat IgG or goat anti-rabbit IgG, both coupled to Alexa 594, were used (Molecular Probes, Leiden, The Netherlands).

[3H]AVP competition assay. Cells were seeded on 12 multwell filters at a density of 150,000 cells/cm² and grown for 3 days. Cells were subsequently treated with the antagonists for 16 h, followed by three washes with ice-cold phosphate-buffered saline containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS-CM). The cells were then incubated for 1 h on ice with [3H]AVP (PerkinElmer Life Sciences, Boston, MA) and the antagonist diluted in PBS-CM. Cells were washed three times with ice-cold PBS-CM, followed by excision of the filters and counting of the radioactivity as described elsewhere (23). Triplicate samples were measured, and every experiment was performed at least in triplicate.

cAMP measurements. MDCK II cells were seeded on 24 multwell filters at a density of 150,000 cells/cm², grown to confluence, and treated with the antagonists as indicated. Subsequently, cells were briefly washed in PBS-CM, followed by incubation for 10 min in culture medium supplemented with 250 μM IBMX (Sigma) to prevent cAMP degradation by phosphodiesterases. Cells were then challenged for 10 min with DDAVP on the basolateral side in the presence of IBMX. After three washing steps with PBS-CM, cells were lysed in 100 μl of 0.1 M HCL, and cAMP was measured using a fluorescent cAMP assay kit (Sigma) according to the manufacturer’s protocol. Triplicate samples were measured, and experiments were performed at least in triplicate.

Statistical method and analysis. The statistical method used was Student’s t-test. Averaged data of more than three independent experiments are shown, and error bars represent SD. Statistical analysis was performed using Microsoft Excel software.

RESULTS

Maturation of V2R mutants on antagonist treatment. During folding in the ER, V2R is expressed in its high-mannose glycosylated form. As it traverses the Golgi compartment on its way to the BM, it matures to complex- and O-glycosylated proteins. We have previously shown that missense V2R-GFP proteins in NDI that are trapped in the ER (class II) do not undergo maturation and are therefore visible as immature proteins of 60–63 kDa when expressed in MDCK I cells (24). Type I cells, however, endogenously express low levels of V2R and are thus not suitable for functional testing of mutant receptors. As MDCK II cells lack V2Rs (20), we stably expressed wild-type (wt) V2R, the functional mutants V2R-L44P, -I130F, and -S167T, and the nonfunctional V2R-S167L in these cells. As found for MDCK I cells, wt-V2R was mainly

![Fig. 1. Maturation of mutant V2 receptor (V2R) on antagonist treatment is compound and mutation specific. Madin-Darby canine kidney (MDCK) II cells expressing either wild-type (wt)-V2R, V2R-L44P, -I130F, -S167T, or -S167L fused to green fluorescent protein (GFP) were seeded on filters and grown to confluence, after which they were treated for 16 h with 1 μM of indicated cell-permeable V2R antagonists. Untreated cells (Untr.) were used as a control (indicated). Cells were lysed and subsequently analyzed by SDS-PAGE followed by immunoblotting using anti-GFP antibodies. Molecular masses (in kDa) are indicated on the left.](https://www.ajprenal.org)
expressed in the mature 75-kDa form, while the missense mutants were present as immature proteins of 60–63 kDa (Fig. 1, untreated samples). In addition, wt-V2R was mainly expressed in the BM, whereas the mutants were trapped in the ER (Fig. 2, untreated cells).

As maturation can serve as a read-out for translocation of mutant receptors to the BM, the cell-permeable antagonists were tested for their ability to induce receptor maturation. As shown in Fig. 1, overnight treatment with 1 μM V1R antagonist SR4(9059) or the V2R antagonists OPC3(1260), OPC4(1061), and SR1(21463) did not significantly (P > 0.05) increase the 75-kDa signal of wt-V2R. Treatment with OPC4 and SR1, however, caused a decrease or complete disappearance of the 60-kDa signals, respectively, suggesting that these compounds somewhat stabilize and increase receptor maturation of wt-V2R. Treatment of MDCK cells expressing V2R-L44P, -I130F, or -S167T (Fig. 1, middle 3 panels) with the four compounds resulted in increased maturation of all receptor mutants. However, the extent of maturation differed, as less matured V2R proteins were observed with SR4 compared with the other compounds. Moreover, especially for SR1, increased receptor maturation was accompanied by a decrease in the 60-kDa signal. Maturation of V2R-S167L, the nonfunctional mutant, was not increased by treatment with any of the compounds tested, although its expression was somewhat increased with SR1 (Fig. 1, bottom). In MDCK type I cells, similar effects on maturation for the four compounds were observed for V2R-L44P, -Δ62–64, -R113W, -I130F, -G201D, -T204N (not shown), and V2R-S167T and -V206D (26).

**Rescue of V2R mutant plasma membrane expression on antagonist treatment.** To determine whether increased receptor maturation coincided with increased BM localization, the cells were also subjected to CLSM analysis. As reported for MDCK type I cells (23), wt-V2R was predominantly present in the BM of untreated MDCK II cells. Its localization was not affected by treatment with any of the compounds (Fig. 2, top row). Without treatment, V2R-L44P, -I130F, -S167T, and -S167L were retained in the ER (Fig. 2 for V2R-L44P, -S167L), where they colocalized with the ER marker protein disulfide isomerase.

Treatment for 16 h with 1 μM SR4 did not visibly change the localization of V2R-L44P (Fig. 2, middle row). Treatment with OPC3, OPC4, or SR1, however, resulted in a clear translocation of V2R-L44P to the BM (Fig. 2, middle row), after which the localization was similar to that of wt-V2R. V2R-I130F and -S167T proteins responded similarly to the antagonist treatments as -L44P (not shown). V2R-S167L, however, did not translocate to the BM on antagonist treatment but remained trapped in the ER (Fig. 2, bottom row). The lack of a visible translocation of the V2R mutants by SR4, whereas maturation was clearly observed (Fig. 1), indicates that CLSM is less sensitive than immunoblotting.

**Functional rescue of V2R mutants on antagonist treatment.** Following rescue to the plasma membrane, the antagonists need to be displaced by an agonist to have functional rescue. To study the rate of displacement, we used radioactively labeled AVP, as this most closely resembles the natural ligand of the V2R. When untreated, the amount of [3H]AVP bound by mock-transfected cells was low compared with wt-V2R-expressing cells (Fig. 3A). Also, [3H]AVP binding to untreated MDCK II cells expressing V2R-L44P, -I130F, -S167T, or -S167L was not significantly (P > 0.05) different from binding to mock-transfected cells. To further exclude the presence of endogenous V2R in these cells, or the presence of low levels of V2R mutants in the plasma membrane, we determined whether DDAVP induces cAMP generation in these cell lines. However, treatment of mock-transfected MDCK II cells with 100 nM DDAVP did not result in a cAMP response, whereas cells stably expressing wt-V2R showed an ~10-fold increase in intracellular cAMP levels compared with untreated cells. Also, the cell lines expressing the mutants V2R-L44P, -I130F, -S167T, and -S167L did not respond to DDAVP treatment. In addition, basal cAMP levels were not significantly different between all cell lines and clones tested. Together, these data

![Fig. 2. Selective restoration of the plasma membrane expression of V2R-L44P by pharmacological chaperones. MDCK II cells expressing wt-V2R, V2R-L44P, or V2R-S167L (indicated) were seeded, grown, and treated for 16 h with 1 μM of indicated cell-permeable V2R antagonists. Untreated cells were used as a control (indicated). Cells were fixed, subjected to immunocytochemistry to stain for the endoplasmic reticulum (ER) marker protein disulfide isomerase (PDI; indicated in red), and analyzed by confocal laser-scanning microscopy. V2R-GFP is indicated in green.](image-url)
reveal that without rescued cell surface expression of V2R mutants, these cells lack the ability to bind AVP or generate cAMP in response to DDAVP.

This was different with rescued cell surface expression. Pretreatment of MDCK-V2R cells with 1 μM SR4 did not interfere with binding of AVP at all, as a similar amount of AVP was bound as found for nonpretreated control MDCK-V2R cells (Fig. 4A). In contrast, both OPC3 and OPC4 treatment reduced the amount of available binding sites for the wild-type receptor to ~30% of the nonpretreated control MDCK-V2R cells (Fig. 4A). Finally, pretreatment with SR1 decreased the amount of available wt-V2R binding sites by 95% compared with control cells, indicating that this compound is hardly displaced with 100 nM [3H]AVP.

Subsequently, AVP binding was tested on the V2R mutants treated with the antagonists. Although we observed no BM localization, but some maturation, for V2R-L44P, -I130F, or -S167T on treatment with SR4, this compound increased the number of AVP binding sites 4.2-fold (Fig. 4B). OPC3 and

**Fig. 3.** Effect of DDAVP on MDCK cells expressing wt-V2R or mutants in NDI. A: MDCK II-wt-V2R, V2R-L44P, -I130F, -S167T, or -S167L (indicated) were seeded on filters, grown to confluence, washed with ice-cold PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS-CM), and subsequently incubated for 1 h with 100 nM [3H]AVP for 1 h at 4°C to allow radioligand binding to cell-surface receptors. Subsequently, the filters with the cells were washed 3 times with ice-cold PBS-CM, excised, and counted in a scintillation counter. B: MDCK II-wt-V2R, V2R-L44P, -I130F, -S167T, or -S167L (indicated) were seeded on filters, grown to confluence, and subsequently treated with IBMX alone, or in combination with 100 nM DDAVP on the basolateral side. Subsequently, cells were lysed and cAMP accumulation was measured using a fluorescent cAMP assay kit. Triplicate samples were measured, and experiments were performed at least in triplicate. *Significantly different from untreated samples (P < 0.01).

**Fig. 4.** Functional rescue of V2R mutants. MDCK II-wt-V2R (A) or V2R-L44P, -I130F, -S167T, or -S167L (indicated; B) cells were seeded on filters, grown to confluence followed by 16 h treatment with 1 μM of the indicated pharmacological chaperones or left untreated. Next, cells were washed with PBS-CM followed by labeling with 100 nM [3H]AVP for 1 h at 4°C. Filters were washed 3 times with PBS-CM, excised, and counted in a scintillation counter. Values for untreated samples were set to 1. C: MDCK II-wt-V2R, MDCK-V2R-L44P, -I130F, -S167T, or -S167L cells (indicated) were seeded, grown, and treated as described above. Cells were subsequently washed in PBS-CM and challenged for 10 min with 100 nM DDAVP in the presence of IBMX. cAMP accumulation was measured using a fluorescent cAMP assay kit. All experiments were performed at least in triplicate.
OPC4 treatment, which clearly increased V2R mutant BM localization and maturation, increased the number of binding sites for these three mutants two- to threefold. In contrast, despite the clear BM localization and maturation of V2R-L44P, -I130F, or -S167T on treatment with SR1, incubation with this drug did not lead to a significant increase in binding sites for these mutants ($P > 0.05$, $n = 3$; Fig. 4B). No significantly increased numbers of AVP binding sites were measured for the nonfunctional mutant V2R-S167L with any of the treatments (Fig. 4B).

To test whether AVP binding also leads to intracellular signaling, cAMP measurements were performed following the same treatments as for the binding experiments. The relative cAMP levels generated (Fig. 4C) were in line with the obtained levels of AVP binding (Fig. 4B).

**Functional rescue at reduced antagonist and AVP concentrations.** In line with the choice for the use of a V1R antagonist in patients (5), our data above suggest that SR4 is most effective to functionally rescue mutant V2R in patients. However, the concentrations of the antagonists (1 μM) and AVP (100 nM) used will be difficult to obtain in patients. Therefore, we tested functional rescue of the V2R mutants at decreased antagonist concentrations and measured cAMP levels after stimulation with 0.1–10 nM concentrations of AVP. As shown in Fig. 5A, pretreatment of V2R-L44P-expressing cells with 1 or 0.1 nM SR4 did not yield a further cAMP response on stimulation with any of the AVP concentrations used. Pretreatment with 1 or 0.1 nM OPC3 or OPC4, however, led to a two- to fourfold increase in cAMP levels when cells were stimulated with 1 or 10 nM AVP, respectively. Similar results were obtained for V2R-I130F and -S167T (not shown). Pretreatment with SR1 did not result in significantly increased cAMP levels ($P > 0.05$) when tested in the conditions above (not shown).

To determine the level of rescued cell surface expression at lower CPA concentrations, cells expressing V2R-L44P, -I130F, -S167T were treated for 16 h with 100–3 nM concentrations of OPC3, OPC4, or SR1 and immunoblotted (Fig. 5B). At 100 and 30 nM concentrations, the extent of maturation (75–80% of band intensity) of V2R-L44P was highest for SR1 and OPC4, whereas OPC3 showed a reduced level of maturation (60–70%) only (not shown). Treatment with SR1 did not result in significantly increased cAMP levels ($P > 0.05$) when tested in the conditions above (not shown).

**Time-resolved functional rescue of V2R mutants.** On administration to patients, the blood concentrations of the antagonists will not be stable in time. Therefore, it is important to know how long it takes for the antagonists to confer a functionally rescued V2R phenotype, and whether this is different between antagonists. To study this, cells expressing V2R-L44P, -I130F, and -S167T were treated at different time points with 0.1 nM of the pharmacological chaperones, followed by [3H]AVP labeling to semiquantify the available AVP binding sites at the cell surface. Treatment of V2R-L44P, -I130F, or -S167T cells with OPC3 and OPC4 increased the available binding sites up to threefold, which became apparent after 8 h of treatment and did not further increase between 8 and 16 h of treatment (Fig. 6). Consistent with the absence of any rescue at the low concentrations used, SR4 treatment did not significantly ($P > 0.05$) affect [3H]AVP binding at any of the time points. These data indicated that in cell culture, between 4 and 8 h of treatment with a 0.1 nM antagonist is needed for a maximal functional rescue of the V2R mutants.

**DISCUSSION**

Pharmacological chaperones rescue a broad spectrum of V2R mutants. By definition, class II mutant proteins are ER retained due to misfolding. Binding of an antagonist to a mutant receptor can reverse the distorting effect of the mutation and thus aid in protein folding (3, 32). Indeed, our study reveals that all nine V2R mutants in NDI tested, except for V2R-S167L, are stabilized by the antagonists used, resulting in different levels of receptor maturation. In line with the finding of Tan et al. (31) that achieving the proper complex glycosylation state is necessary for V2R to reach the BM, maturation of the V2R mutants on immunoblotting coincides with BM ex-
expression as detected by CLSM. Consistently, V2R-S167L did not mature with any of the ligands and failed to leave the ER, which has been suggested to be due to severe distortion of the structure (34). Our data indicate, however, that V2R-S167L can be bound by the V2R antagonist SR1, as administration of this drug increased the amount of immature proteins (Fig. 1). A similar ER-stabilizing effect has been observed for the non-peptide antagonist naltrexone on immature forms of the opioid receptor (21).

In line with the data from Morello et al. (18), the rescue of multiple V2R mutants reveals the high efficacy by which nonpeptide antagonists stabilize ER-retained mutant proteins. These effects were observed for different clones obtained in MDCK I (not shown) and MDCK II cell lines, indicating that the effects were inherent to the V2R mutant and not due to clonal differences. In contrast, using the same V2R mutants, a subset of chemical chaperones restored the BM localization of only one mutant, V2R-V206D (26). In addition, the level of maturation and cell surface rescue of V2R-V206D on treatment with chemical chaperones was also less than observed for the antagonists OPC3, OPC4, and SR1 (not shown). This is likely due to their different modes of action, as cell-permeable antagonists stabilize the receptor’s conformation through direct interaction, whereas chemical chaperones may evoke a stress response, modify the activity of folding proteins, or dehydrate the mutant’s environment (33).

In general, the extent of cell surface rescue of V2R mutants is determined by the affinity of the antagonists. Brothers et al. (6) suggested that COOH-terminal fusion of GFP to a receptor might induce plasma membrane expression. However, our data reveal that this is not the case here, as the MDCK cells expressing the GFP-tagged V2R mutants have no significant radioligand binding or cAMP signaling after agonist stimulation (Fig. 3). Following rescue to the cell surface, however, most of the V2R mutants are able to bind AVP and consequently elicit a cAMP response. Our data show that SR1 and OPC4 induce maturation of V2R-L44P, -I130F, and -S167T the best, followed by OPC3 and, much less, SR4 (Figs. 1 and 5B). Similarly, at 1 μM concentrations, SR1, OPC4, and OPC3 induce a robust cell surface rescue, whereas SR4 induced no detectable translocation (Fig. 2). A similarly reduced rescue for V2R-S167T and V2R-ΔΔ2–64 by SR4 compared with SR1 was found by others (34). With the exclusion of severely distorted receptors (V2R-S167L), our data indicate that the level of maturation and translocation of V2R mutants in general is directly related to the antagonists’ affinities for V2R (Table 1), as similar relative effects were observed for eight of nine V2R mutants tested.

Functional rescue is a balance between membrane expression and displacement by DDAVP. Once at the plasma membrane, functional rescue can only occur if the pharmacological chaperone is displaced by an agonist, thereby allowing receptor activation and induction of the signaling cascade (12). Our data reveal that for functional rescue of V2R mutants, the pharmacological chaperone should fit two contradictory criteria: it should have a sufficient high affinity to facilitate the mutant receptor’s stabilization and translocation to the plasma membrane, but its binding should not be so strong as to interfere with its displacement by AVP. Moreover, our data confirm that the extent of functional rescue critically depends on the concentration used (12, 18).

At high concentrations (1 μM), SR4 showed a weak cell surface rescuing effect for the V2R mutants, whereas OPC3, OPC4, and SR1 rescued large amounts of receptor to the BM (Figs. 1 and 2). Nevertheless, subsequent AVP binding and cAMP generation are considerably lower for OPC3-, OPC4-, and SR1- compared with SR4-treated cells and correlates largely with their affinities for the V2R (Fig. 4, B and C; Table 1). These data indicated that the extent of displacement by DDAVP is of major importance at these concentrations. A better functional rescue is obtained with a few rescued receptors which are fully available for AVP binding (SR4) than when many V2R mutants are rescued, which are limitedly available for AVP binding (OPC3, OPC4, SR1). At low concentrations, however, pretreatment with (sub)nanomolar concentrations of OPC3, OPC4, or SR4, followed by stimulation with 1–10 nM AVP, only resulted in increased cAMP levels when many V2R mutants are rescued, which are limitedly available for AVP binding (OPC3, OPC4, SR1). As shown in Fig. 5B, low concentrations of OPC3 and OPC4 are still able to induce cell surface trafficking and maturation, whereas SR4 is not. Therefore, these effects were observed for eight of nine V2R mutants tested.

Table 1. Characteristics of pharmacological chaperones

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Kᵢ, nM</th>
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<tr>
<td>SR4095</td>
<td>SR4</td>
<td>275±50</td>
</tr>
<tr>
<td>OPC31260</td>
<td>OPC3</td>
<td>9.42±0.90</td>
</tr>
<tr>
<td>OPC41061</td>
<td>OPC4</td>
<td>0.43±0.06</td>
</tr>
<tr>
<td>SR121463</td>
<td>SR1</td>
<td>0.54±0.08</td>
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</table>

Values are means ± SE. V1R and V2R, V1 and V2 receptor, respectively. The values of the inhibitory constant (Kᵢ) for OPC3(1260), OPC4(1061), SR4(09059), and SR1(21463) on the human V2R are as described elsewhere (28–30, 36).
fore, at low antagonist concentrations, the extent of rescued cell surface expression becomes critical.

The absence of functional rescue on SR1 treatment seems to contradict data published by Morello et al. (18), who found that cAMP levels increased up to 15-fold in V2R mutants pretreated with SR1. This difference is most likely due to the higher agonist/antagonist ratio and concentrations used by Morello et al., which were 10- and 100-fold higher compared with our “high concentration amounts,” respectively. This, however, provides additional support that the observed effect on cAMP generation depends on the concentrations used and ratios of antagonist to agonist.

Surprisingly, OPC4 and SR1 have similar affinities for the V2R (Table 1), but OPC4 was easier displaced by AVP than SR1 (Fig. 4A) and consequently yielded better functional rescue at any concentration used (Fig. 4, B and C). This difference was not caused by a reduced V2R mutant cell surface expression with SR1, as at low concentrations this was similar to, or better than, that of OPC4 or OPC3. Possibly, the different effects observed for SR1 and OPC4 might be due to differences in their V2R binding sites, as recently established (15). This is underscored by our finding that SR1, but not OPC4, stabilizes the ER-retained form of V2R-S167L (Fig. 1). These data reveal that compound-intrinsic factors other than their affinities influence their extent of displacement by AVP and ability to confer functional rescue.

**Optimal pharmacological chaperone to treat congenital NDI.** Treatment with SR4 showed a significant increase in urine concentration in three NDI patients encoding V2R-R137H, thereby providing proof of the principle that pharmacological chaperones can relieve NDI (5). In two other patients encoding V2R-W164S and Δ62–64 (185–193del), however, the response to SR4 treatment was weaker. Interestingly, V2R-R137H is only a partial class II mutant, as a considerable portion of this mutant is fully matured but is constitutively internalized from the plasma membrane (class V) (1), whereas V2R-W164S and Δ62–64 are fully retained in the ER (5). The reduced ER retention suggests a low level of misfolding of V2R-R137H, and the difference in the extent of ER-retention between V2R-R137H and other V2R mutants may underlie the observed effects of SR4 in NDI patients (4). Likely due to its low maximal blood plasma concentration of 30 nM (D. Bichet, personal communication), SR4 does not effectively rescue full class II mutants at low concentrations (Fig. 5A). As OPC3 and OPC4 allow functional rescue of fully ER-retained V2R mutants at nanomolar concentrations and NDI patients harboring full class II mutations are much more common (25), these compounds are anticipated to relieve NDI better than SR4 and in more NDI patients. Moreover, and in line with the adopted strategy by Bernier et al. (5), continuously elevated levels of the antagonists are needed, as it takes >4 h before a functional rescue is obtained (Fig. 6). Since nonpeptide antagonists remain active in vivo up to 8 h (11, 27), this would require the administration of at least three doses per day. The analyses in patients will be the subject of future studies.

In conclusion, we have demonstrated that cell-permeable V2R antagonists can rescue the cell surface expression of a broad spectrum of ER-retained V2R mutants and that functional rescue is a balance between a cell-permeable antagonist’s ability to rescue the cell surface expression of the V2R mutant and its ability to be displaced by AVP. Moreover, we show that at low concentrations the functional rescue occurs most efficiently by antagonists with a relatively high affinity for the receptor (Fig. 7). Our findings that a large number of V2R mutants are rescued by pharmacological chaperones and that functional rescue of mutant V2Rs at low antagonist concentrations is most effective with relatively high-affinity antagonists are anticipated to become important for other diseases, such as hypogonadotrophic hypogonadism (12), early-onset obesity (16), or hypothyroidism (7), in which mutations in G protein-coupled receptors are causal and for which cell-permeable antagonists are, or may become, available.

Concerning NDI patients with V2R mutations, of the four compounds tested, OPC3(1260) and OPC4(1061) combine cell surface rescue and displacement by AVP best when tested with low antagonist and near-physiological AVP concentrations. While other high-affinity V2R antagonists might be as suitable, OPC31260 is currently being tested as a treatment for polycystic kidney disease (2), while OPC41601 is under trial to treat hyponatremia and congestive heart failure in humans (19). Since negative side or toxicity effects have not been reported in these studies, OPC31260 and OPC41061 represent safe and promising candidates to treat NDI in patients with type II mutations in the V2R. (30, 36)

**ACKNOWLEDGMENTS**

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