Autosomal dominant polycystic kidney disease (ADPKD) is associated with progressive enlargement of cysts, leading to a decline in function and renal failure that cannot be prevented by current treatments. Mutations in \( \text{pkd1} \) and \( \text{pkd2} \), encoding the polycystin 1 and 2 proteins, induce growth-related pathways, including heat shock proteins, as occurs in some cancers, raising the prospect that pharmaceutical interventions that target these pathways might alleviate or prevent ADPKD. Here, we demonstrate a role for VX-809, a corrector of cystic fibrosis transmembrane conductance regulator (CFTR), conventionally used to manage cystic fibrosis in reducing renal cyst growth. VX-809 reduced cyst growth in \( \text{Pkd1}^{-/-} \)-knockout mice and in proximal, tubule-derived, cultured \( \text{Pkd1} \) knockout cells. VX-809 reduced both basal and forskolin-activated AMP levels and also decreased the expression of the adenyl cyclase AC3 but not of AC6. VX-809 also decreased resting levels of intracellular \( \text{Ca}^{2+} \) but did not affect ATP-stimulated \( \text{Ca}^{2+} \) release. Notably, VX-809 dramatically decreased thapsigargin-induced release of \( \text{Ca}^{2+} \) from the endoplasmic reticulum (ER). VX-809 also reduced the levels of heat shock proteins Hsp27, Hsp70, and Hsp90 in mice cystic kidneys, consistent with the restoration of cellular proteostasis. Moreover, VX-809 strongly decreased an ER stress marker, the GADD153 protein, and cell proliferation but had only a small effect on apoptosis. Given that administration of VX-809 is safe, this drug potentially offers a new way to treat patients with ADPKD.

Autosomal dominant polycystic kidney disease (ADPKD)\(^2\) \(1\) is characterized by multiple renal cysts, hypertension, and a decline in renal function that culminates in renal failure in 50% of all ADPKD patients \(2\). Two proteins, PC1 and PC2, are associated with ADPKD. Mutations in \( \text{pkd1} \) produce 85% of the cases of ADPKD \(2, 3\). Patients with \( \text{pkd2} \) mutations have a similar disease that is considered milder because of its later onset of symptoms \(4\).

Cysts can develop in each nephron segment through a combination of aberrant epithelial cell proliferation and abnormal fluid secretion \(5\). Cystic fluid is produced by a cAMP-dependent mechanism similar to that found in secretory epithelia \(6\). Elevated cAMP levels have been detected in animal models of ADPKD, and this cAMP elevation provides the stimulation for fluid secretion in the cysts. An important step in cAMP-dependent fluid secretion is the activation of the cystic fibrosis transmembrane conductance regulator (CFTR), which secretes chloride into the cyst lumen followed by sodium and water \(7\).

CFTR is a cAMP-dependent chloride channel \(8\) best known for its role in cystic fibrosis (CF) \(9\). The most common mutation is \( \Delta F508 \)-CFTR, found in the nucleotide-binding domain 1 (NBD1) region of the CFTR molecule; this mutation affects about 90% of all CF patients \(10\). Correctors have been identified that act on \( \Delta F508 \)-CFTR either directly or indirectly to attenuate the deleterious effects of the disease \(11\). One of these correctors that has is currently approved for use in patients, namely VX-809, restores the trafficking of \( \Delta F508 \)-CFTR to the plasma membrane \(12\). Precisely how VX-809 rescues CFTR function is still unclear. Some researchers maintain that VX-809 acts directly on CFTR, particularly within the membrane-spanning domain 1 of transmembrane domain 1 \(13\). We have shown that corrector C18, a close relative of VX-809 \(14\), in combination with corrector C4 \(11\), alters a proteostatic network of proteins \(15\) to rescue CFTR \(16\).

Heat shock proteins are abnormally regulated in ADPKD cysts \(17\), indicating the occurrence of a heat shock response \(18\) in proliferating cyst cells. Indeed, the down-regulation of Hsp90 reduces cyst growth \(17\). Along these same lines, we provide here a novel strategy based on the use of VX-809 to reduce cyst growth in ADPKD. We found that treatment of cysts with VX-809 reduces their growth by restoring a network of proteostatic proteins that, when misregulated, contribute to cyst growth in ADPKD.

**Results**

**VX-809 reduces cyst growth and improves renal function in \( \text{Pkd1}^{\text{fl/fl}};\text{Pax8}^{\text{GTA}};\text{TetO-cre} \) mice**

To show that VX-809 is effective in reducing cyst growth \textit{in vivo}, we injected the drug into the intraperitoneal (IP) space in \( \text{Pkd1}^{\text{fl/fl}};\text{Pax8}^{\text{GTA}};\text{TetO-cre} \) model mice. When treated with doxycycline, these mice express Cre, causing knockout of PC1 \(19\). As has shown previously, an injection of doxycycline...
results in the development of multiple large cysts in these mice at ~3 weeks of age (19, 20) (Fig. 1A). In sharp contrast, when this strain of mice was injected daily with VX-809 (30 mg/kg) from postnatal day (PND) 10 to PND20, they showed significantly less cyst growth (~60.4%) (Fig. 1, A and B). Kidney weight (Fig. 1C) and kidney-to-body weight ratios (Fig. 1D) were also lower than those of the control mice. There was no difference in overall body weight (Fig. 1E) between the treated and untreated groups. The administration of VX-809 improved renal function, as evidenced by lower blood urea nitrogen (BUN) (F) and creatinine (G) levels. The total kidney area and total cystic area were measured with ImageJ (provided by National Institutes of Health). Cystic index = 100 × (total cystic area/total kidney area) and is expressed as a percentage. Columns represent mean ± S.E. for DMSO (vehicle)-treated (n = 4–5) and VX-809–treated mice. *, p < 0.05; **, p < 0.01 (for all graphs). Statistical analysis was performed using an unpaired two-tailed Student’s t test.

**VX-809 reduces cyst growth in vitro**

We conducted our experiments using a model ADPKD cell line (PH, pkd1+/− heterozygote control; PN, pkd−/− knockout or Pkd1-null) clonally isolated from single parental clones obtained from a pkdfl mouse that was manufactured in an ImmortoMouse containing the H-2Kb-tsA58 gene. The null cells (PN) stably express the Cre recombinase. All of the cells are of proximal tubule origin (21, 22).

One of the key questions we addressed was how VX-809 reduces cyst growth. For this purpose, we grew cysts in the presence of forskolin (Fig. 2). After treatment with forskolin, the cysts obtained were larger than those in the control cells, indicating that cyst growth is indeed cAMP-dependent as shown previously (23). Fig. 2 shows the effect on cyst growth of C18 or VX-809 when administered every other day, as well as the same treatment administered from days 9–16 in mice with already established cysts. Importantly, this drug treatment regime was effective in dramatically reducing cyst size, even in the presence of the hyperstimulatory environment created by forskolin. Also, VX-809 and a related compound, C18 (16), were both able to reduce cyst growth equally well in the presence and absence of forskolin (Fig. 2). This important finding shows that VX-809 can overcome the powerful stimulatory effect of forskolin-induced increases in cAMP levels and still reduce cyst growth. Taken together with the animal data3 provided in Fig. 1, these in vitro results clearly indicate that CFTR correctors are effective in reducing cyst growth.

**VX-809 reduces proliferation**

A hallmark of the cysts in ADPKD is the increase in proliferation in response to cAMP (24). Therefore, we asked whether VX-809 would affect proliferation. We found that administration of VX-809 at 30 mg/kg (see Fig. 1) to mice (Fig. 3, A–C) or to cells (Fig. 3D) at 10 μM did significantly inhibit cell proliferation when compared with that of DMSO-treated mice or cells.

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3 All data generated or analyzed during this study are included in this published article and are available from the corresponding author upon reasonable request.
The corrector VX-809 slows cyst growth in ADPKD

VX-809 down-regulates cAMP levels

We have shown previously in both animal and cell culture models of ADPKD that cells lacking functional PC1 have elevated cAMP levels when compared with those that contain functional PC1 (25). To assess the effect of CFTR correctors, we measured cAMP activity in PC1-conditional knockout mouse kidneys and in PN cells that were either left untreated or treated with VX-809. We found that administration of VX-809 (Fig. 4, A and B) significantly decreased the cAMP levels. Fig. 4B shows that PH (pkd1 heterozygote) cells have lower resting cAMP compared with PN cells, as we also have shown in previous findings (26). Next, we treated the cells with forskolin, which increased cAMP levels drastically, as expected (Fig. 4B). It should be noted that VX-809 reduced the forskolin-induced increase in cAMP, indicating a direct action of VX-809 on adenylyl cyclase activity. To determine whether VX-809 also inhibits phosphodiesterase activity, we treated the cells with forskolin or with forskolin in combination with 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, to maximally stimulate adenylyl cyclase activity. Treating PN cells with forskolin or IBMX (Fig. 4B) increased the cAMP activity by ~10-fold. However, VX-809 did not affect the forskolin- or IBMX-induced increases in adenylyl cyclase activity (Fig. 4B, compare data bars 5 and 9), indicating that VX-809 does not affect phosphodiesterase activity. These data suggest that under basal conditions, one way that VX-809 reduces cyst growth (as shown in Fig. 1) is most likely by reducing the resting cAMP levels. However, the results presented above, indicating that VX-809 can inhibit cyst growth even in the presence of forskolin (which elevates cAMP activity almost 500-fold), strongly suggest that other mechanisms are also involved.

VX-809 reduces AC3 but not AC6

Ca²⁺-dependent adenylyl cyclase (AC) activity has been shown to play a role in cyst growth in ADPKD (27). There are two classes of adenylyl cyclases that are regulated by intracellular Ca²⁺: one class is activated and the other inhibited. Thus, we chose to focus on one member from each of these two classes: AC3 and AC6. AC6 activity is inhibited by Ca²⁺, whereas that of AC3 is enhanced by increases in intracellular Ca²⁺ (28). Both AC3 and AC6 are expressed in the proximal tubules of rat kidneys (29), and AC6 is already known to play a role in ADPKD (28). We have now shown (Fig. 4, C–E) that AC3 and AC6 are both expressed in PN cells. Surprisingly, the AC3 levels were decreased following treatment of the PN cells with VX-809. This result is consistent with our observation that VX-809 reduces both basal and forskolin-stimulated cAMP levels.

VX-809 down-regulates resting intracellular Ca²⁺ levels and release of Ca²⁺ from the ER

Misregulation of Ca²⁺-dependent signal transduction is associated with cyst formation (27, 30). To determine whether VX-809 alters Ca²⁺ movement, we treated the cells with ATP, which stimulates purinergic receptors (31) and found (Fig. 5, A–C) that VX-809 caused a small reduction in resting Ca²⁺ but had no effect on intracellular Ca²⁺ movement in response to ATP. We suggested previously that PC2 is a positive regulator of ER Ca²⁺ release (32, 33). To address the effect of VX-809 on
this Ca²⁺ release, we treated the cells with thapsigargin, a specific inhibitor of the ER Ca²⁺-ATPase which, when applied, allows Ca²⁺ to leak out of the ER through independent Ca²⁺-permeable pathways (34). Again, we saw a small effect of VX-809 on resting Ca²⁺. Importantly, VX-809 dramatically reduced the thapsigargin-induced release of Ca²⁺ from the ER (Fig. 5, D–F).

**VX-809 has no effect on PC2 but increases CFTR expression**

PC2 is a 968–amino acid protein with an approximate molecular mass of 100 kDa in its monomeric form. To determine whether VX-809 affects PC2 protein expression, we conducted Western blotting experiments on PN cells to assess the resting levels of endogenous PC2. Our results indicated (Fig. 6, A and B) that VX-809 has no effect on PC2 expression.

Fig. 6, C and D, shows that VX-809 did significantly increase the levels of CFTR in PN cells. It is known that the processing of WT CFTR is increased following CFTR corrector treatment by increasing its processing (16).

**VX-809 reduces the steady-state levels of heat shock proteins**

To facilitate their growth in the kidney, cysts develop an altered network of proteins that most likely serves to protect them from stress (35). Thus, one may ask whether restoring this network to its normal state would reduce cyst growth. We have shown previously that CFTR-based correctors rescue ΔF508-CFTR by altering the proteostatic network of proteins involved in its degradation (16). Therefore, we asked whether VX-809 would alter the levels of Hsp in pkd¹⁻/⁻ mice and in PN cells. Fig. 7 shows the results in mice. The first thing to note is that Hsp27, -70, and -90 are all elevated in cystic kidneys compared with normal controls. Treatment with VX-809 of kidneys in which PC1 levels had been knocked out resulted in significantly less expression of Hsp27, Hsp70, and Hsp90. There was no change in Hsp40. Likewise, PN cells treated with VX-809 (Fig. 8, A–D) showed reduced expression of Hsp27, Hsp70, and Hsp90 following treatment. Both sets of data are consistent with an alteration in the heat shock response to cyst growth. A comparison of the PH cells, which are pkd¹⁻/⁻, with the PN cells, which are pkd⁻¹⁻, shows that Hsp27 and -90 are all elevated in cystic kidneys compared with normal controls. Treatment with VX-809 of kidneys in which PC1 levels had been knocked out resulted in significantly less expression of Hsp27, Hsp70, and Hsp90. There was no change in Hsp40. Likewise, PN cells treated with VX-809 (Fig. 8, F–H) showed reduced expression of Hsp27, Hsp70, and Hsp90 following VX-809 treatment. Both sets of data are consistent with an alteration in the heat shock response to cyst growth. A comparison of the PH cells, which are pkd¹⁻/⁻, with the PN cells, which are pkd⁻¹⁻, shows that Hsp27 and -90 are all elevated in cystic kidneys compared with normal controls. Treatment with VX-809 of kidneys in which PC1 levels had been knocked out resulted in significantly less expression of Hsp27, Hsp70, and Hsp90. There was no change in Hsp40. Likewise, PN cells treated with VX-809 (Fig. 8, A–D) showed reduced expression of Hsp27, Hsp70, and Hsp90 following treatment. Both sets of data are consistent with an alteration in the heat shock response to cyst growth. A comparison of the PH cells, which are pkd¹⁻/⁻, with the PN cells, which are pkd⁻¹⁻, shows that Hsp27 and -90 are all elevated in cystic kidneys compared with normal controls. Treatment with VX-809 of kidneys in which PC1 levels had been knocked out resulted in significantly less expression of Hsp27, Hsp70, and Hsp90. There was no change in Hsp40. Likewise, PN cells treated with VX-809 (Fig. 8, A–D) showed reduced expression of Hsp27, Hsp70, and Hsp90 following treatment. Both sets of data are consistent with an alteration in the heat shock response to cyst growth.
shown in Fig. 8C. Thus, although there are some differences between cell and animal models regarding the resting levels of Hsp70, both models show a reduction in Hsp70 when treated with VX-809.

**VX-809 enhances the disappearance of Hsp70 and Hsp90**

To gain more insight into the mechanism whereby VX-809 alters Hsp protein levels, we inhibited translation by using cycloheximide (36) and monitored the disappearance of the three Hsp. Fig. 9, A–D, clearly shows that the steady-state levels of each of the Hsp was unchanged over the 8 h immediately following cycloheximide treatment, suggesting that they are long-lived, stable proteins. In contrast, after treatment with VX-809, the Hsp70 levels dropped over the 8-h period to approximately half the level observed at time 0. Hsp90 was reduced by ∼25%, and Hsp27 was unchanged. These data suggest that VX-809 reduces the half-life of two key Hsp, most likely by increasing their rates of degradation.

**VX-809 has a small effect on apoptosis**

Apoptosis is associated with the absence of PC1 in ADPKD (37). To assess whether apoptosis is altered directly by VX-809, we monitored the caspase-3 activity in PN cells. Fig. 10 shows that there was a small but significant reduction in caspase-3 activity in the PN cells after treatment with VX-809 indicative of a small effect on apoptosis.

**VX-809 dramatically reduces the ER stress-related protein GADD153**

Given that VX-809 alters an ensemble of heat shock proteins, we asked whether it also alters proteins associated with ER stress. To address this question, we measured the levels of three ER stress-associated proteins, 78-kDa glucose-regulated protein (GRP78), ER oxidoreductin 1 (Ero1), and DNA damage-inducible protein 3 (GADD153), also known as C/EBP homologous protein (CHOP) (38, 39). The results depicted in Fig. 11 indicate no changes in GRP78 or Ero1 in response to VX-809. In sharp contrast, there was a dramatic increase in GADD153 when cysts were induced in the mice compared with their normal littermates and an equally dramatic reduction in GADD153 reduction when the cyst containing mice were treated with VX-809. Immunostaining for GADD153 in the mice kidneys also shows a strong reduction when mice are treated with VX-809 (Fig. 12).
The corrector VX-809 slows cyst growth in ADPKD

Ca²⁺ Measurements

A. Ca²⁺ (Δ f/f)

B. Resting Ca²⁺

C. Spike amplitude

D. Ca²⁺ (Δ f/f)

E. Resting Ca²⁺

F. Spike amplitude

![Figure 5. Intracellular Ca²⁺ and ER Ca²⁺ are affected. A, representative traces of intracellular Ca²⁺ release in response to ATP (100 µM) in PN cells and cells treated with VX-809 (10 µM). B and C, graphs summarizing resting calcium levels (B) and the average amplitude of Ca²⁺ release (C) in response to ATP. D, representative traces of ER Ca²⁺ release in response to thapsigargin (4 µM) in PN cells treated with VX-809. E and F, resting calcium levels (E) and the average amplitude of Ca²⁺ release (F) in response to thapsigargin. (F340/F380) levels obtained by ratiometric Fura-2 AM analysis of PN cells treated with VX-809 (10 µM) for 16 h. Amplitude was measured as the standard deviation of the signal base to peak Δf/f. Significance between the two groups was analyzed using Student’s t-test (n = 4–5). *, p < 0.05; ***, p < 0.001.]

Discussion

CFTR and cyst growth

Here we report the surprising finding that VX-809, the drug designed to rescue ΔF508-CFTR in CF, also inhibits cyst growth in ADPKD both in vivo and in vitro. The CFTR protein is found in the apical membrane in the cysts of CF, so treatment with VX-809 might be expected to increase cyst size in ADPKD. Thus, the opposite result is particularly remarkable; in similar in vivo mouse studies in which tubacin was injected IP using a protocol identical to that described in Fig. 1, the tubacin treatment reduced the cyst index by ~15% compared with a 65% reduction following VX-809 treatment. How can this dual function be explained? The effective reduction in cyst index in the mice and in vitro in the PN cells occurred through a series of factors that work together to reduce cyst growth. The inhibition of cyst growth both in vivo in the mouse and in vitro in the PN cells suggests that the CFTR correctors have a direct effect on the cysts rather than having an overall global effect on mouse physiology.

CFTR correctors

High-throughput screening has identified CFTR correctors that can rescue the folding and trafficking of CFTR (12). Correctors also increase the steady-state levels of WT CFTR protein and increase CFTR-generated chloride currents when administered to airway cells (16). Relevant to the present study is the mechanism by which the correctors rescue the trafficking mutants of CFTR. Some investigators have suggested that VX-809 may bind directly to CFTR (40) and have its major effect on membrane-spanning domain (MSD) 1, thereby enhancing the interactions between NBD1 and MSD1 and improving the interaction between NBD1 and MSD2 (13). Others have suggested that misfolded CFTR causes an unfolded protein response in the cell that unleashes a storm of heat shock proteins (41) and that modifying this effect in the direction of normal levels promotes CFTR folding (42). Along these lines, we have shown previously that a combination of correctors, namely C18 + C4, rescues ΔF508-CFTR by decreasing the binding of CFTR to Hsp27 and Hsp40 (16). We took this result further by showing that silencing Hsp27 or Hsp40 has the same effect as applying correctors, suggesting that the correctors alter the interaction between CFTR and the cell’s quality control machinery rather than directly affecting CFTR. Given this background information, it does not seem counterintuitive for VX-809 to reduce cyst growth by restoring cellular proteostasis (15).
The corrector VX-809 slows cyst growth in ADPKD

**Figure 6.** PC2 is unchanged and CFTR expression is increased. A, Western blotting showing expression of PC2 in treated or control cells. B, columns represent the means ± S.E. of the PC2 expression. The data were analyzed by nonparametric t test. The experiment was repeated six (control) or seven times. Note that PC2 levels are unchanged after VX-809 treatment. C, Western blotting showing expression of CFTR in VX-809 treated or control PN cells. D, columns represent averages ± S.E. of the CFTR expression. Data were analyzed by nonparametric t test. Experiment was repeated 4–5 times. **, p < 0.01. Note that VX-809 treatment enhances the CFTR expression in PN cells compared with untreated control cells.

**Figure 7.** Chaperone expression is altered in mice. A and B, representative Western blot images of HSP27, -70, -90, and -40 in lysates of kidney tissue from no cyst induced (ND), cyst induced with doxycycline (D), or cyst-induced pkd/ mice treated with VX-809 (D + VX809) 30 mg/kg body weight. C–F, columns represent averages ± S.E. of HSP27, -70, -90, and -40 expression. Data were analyzed by nonparametric t test. n = 4 for each treated and control group. Vertical black lines between the Western blot lanes are representative of experiments from the same gel. *, p < 0.05; **, p < 0.01. Although lines appear between some of the lanes, the data were extracted from the same gel.
The corrector VX-809 slows cyst growth in ADPKD

VX-809 alters Hsp levels

It is well known that many signal transduction and transcription factor pathways are altered in cystic kidneys as compared with normal kidneys. Recent global gene profiling has indicated a down-regulation of epithelium-associated genes and an up-regulation of genes involved in development, mitogen-activated proliferation, cell cycle progression, epithelial–mesenchymal progression, hypoxia, aging, and the inflammatory response (43). We propose that some of these altered gene profiles are a direct effect of the loss of polycystin (PC) protein function, and others result from an adaptation of the cyst cells to growth in an abnormal and diseased renal environment. For example, the up-regulation of Hsp in cancer cells contributes to the folding of overexpressed proteins, and these Hsp are targets for anticancer drugs (44).

Along these lines, Hsp90 is overactive in ADPKD cells, and reducing its function decreases cyst growth (17, 45). Seeger-Nukpezah et al. (17) have shown that the application of the small-molecule inhibitor STA-2842 over a 10-week period inhibits the initial formation of cysts and also slows the progression of pre-existing cysts, whereas Hsp70 is higher in PH versus PC1 cells, whereas Hsp70 is higher in PH versus PN cells. Data were analyzed by nonparametric t test. The experiment was repeated 4–5 times. Cells were grown in 10-cm culture dishes under permissive conditions (33 °C) with γ-interferon in the culture medium. Cells were then transferred to nonpermissive conditions at 37 °C in γ-interferon–free culture medium and evaluated at full confluence. At day 4, cells were treated with VX-809 (10 μM) for 16 h and harvested on the fifth day for assay. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

VX-809 decreases Hsp levels

Along these lines, Hsp90 is overactive in ADPKD cells, and reducing its function decreases cyst growth (17, 45). Seeger-Nukpezah et al. (17) have shown that the application of the small-molecule inhibitor STA-2842 over a 10-week period inhibits the initial formation of cysts and also slows the progression of pre-existing cysts, not unlike the situation we observed for VX-809. Based on their studies, the VX-809–induced decrease in Hsp90 levels that we noted here would certainly have a negative influence on cyst growth. We have now shown that VX-809 reduces Hsp90 levels in the kidneys of PC1 conditional knockout mice and in PN cells. Both of these observations suggest that VX-809 has a major effect on the protein levels of Hsp90.

In contrast to Hsp90, little is known about the role of Hsp70 or Hsp27 in ADPKD cyst growth. Stress-inducible Hsp70 (HSPA1A) has an ATP-dependent mechanism and plays an important role in protein folding. Hsp70 is also up-regulated in cancer cells and is associated with a negative outcome (46, 47). It is known that silencing of the gene encoding HSP70-1 (HSPA1A) is cytotoxic to cancerous but not to normal cells. Hsp70 has been identified as a promising cancer drug target, stimulating intense interest in identifying and characterizing HSP70 inhibitors for cancer therapy (46). Our data here show that VX-809 dramatically reduces Hsp70 in the kidneys of the conditional PC1 knockout mice and in PN cells, most likely by increasing its turnover.

Hsp27 (HspB1), a member of the small heat shock protein family, is thought to guard against protein aggregation during stress (48). In addition to its role as a chaperone and checkpoint for targeting CFTR for degradation, it also plays a role in p38 MAP kinase–dependent inflammation (49). Hsp27 is phosphorylated by p38 MAP kinase, which promotes dimerization of the protein and inhibits its multimerization (50). In many aggressive cancers, Hsp27 promotes drug resistance, metastasis, and poor patient outcomes. The strong protective effect of Hsp27 is mainly a function of its blockade of apoptosis (51). Our data show that VX-809 indeed induces a profound reduction in...
Hsp27 in both the kidneys of the conditional PC1 knockout mice and in PN cells. Clearly, the down-regulation by VX-809 of key heat shock proteins implicated in the growth of cancer cells (Hsp27, Hsp70, and Hsp90) (51) is a key factor in reducing cyst growth in ADPKD.

VX-809 dramatically reduces the protein expression of GADD153

GADD153 is a transcription factor that is well known to be up-regulated in response to genotoxic challenges such as UV radiation. However, it is also induced by nutrient depletion, including glucose deprivation and amino acid starvation (52). We show here that VX-809 dramatically down-regulated GADD153 in the kidneys of PC1-conditional knockout mice. In contrast, two other indicators of ER stress, Ero1 and GRP78, were unchanged in response to VX-809. Ero1 is tightly linked to protein overload in the ER (53). Likewise, GRP78 is a Ca\(^{2+}\)-dependent chaperone associated with the unfolded protein response (54). The observation that VX-809 does not affect these two stress-related proteins suggests that it is not involved in regulating ER stress in our mouse model of ADPKD. Further evidence of this hypothesis is the observation that VX-809 had a small effect on apoptosis, another indicator of ER stress.

GADD153 is often cited as a key regulator of apoptosis (52). One question is why apoptosis was hardly changed in response to VX-809 despite the large decrease in GADD153? The reason is that GADD153 is associated with both cell death and cell survival. This dichotomy of function has been demonstrated nicely in myelinating cells during development in mice overexpressing GADD153. In this model, over-expression of GADD153 did not promote cell death but instead was suggested to be prosurvival through a dynamic regulatory cycle involving the unfolded protein response. The higher expression levels of GADD153 in the cystic mice may be another indicator of the prosurvival pathways that allow cysts to grow in the kidney at the expense of healthy tissue (55, 56). Thus, its down-regulation by VX-809 is likely the result of the reduction in cyst burden.

VX-809 reduces Ca\(^{2+}\) movement out of the ER

Misregulation of Ca\(^{2+}\) is associated with cyst formation in ADPKD (35), with some investigators reporting that disruption of Ca\(^{2+}\) signaling is the primary event that supports increased cyst growth (30). Several studies have shown that a reduction in the function of either PC1 or PC2 leads to dysregulation of Ca\(^{2+}\) signaling (see Ref. 57 for a review). We show here that VX-809 causes a small reduction in resting Ca\(^{2+}\), but it does not increase intracellular Ca\(^{2+}\) in response to ATP application in PN cells. ATP stimulates purinergic receptors that are themselves Ca\(^{2+}\) channels (31), and thus in our study Ca\(^{2+}\) move-
ments via these receptors were not affected by VX-809. In sharp contrast, VX-809 has a profound effect on the thapsigargin-induced release of Ca\(^{2+}\) from the ER. Thapsigargin inhibits the SERCA pump; therefore, the magnitude of the release of Ca\(^{2+}\) from the ER depends on the Ca\(^{2+}\) gradient between the lumen of the ER and the cytoplasm and on the permeability of the ER membrane for Ca\(^{2+}\). Misregulation of Ca\(^{2+}\) with an up-regulation of store-operated Ca\(^{2+}\) entry (SOCE) (58, 59) is also a risk factor for a poor outcome in cancer. Thus, we propose that VX-809’s inhibition of ER Ca\(^{2+}\) release robs ADPKD cysts of the ability to respond to growth stimuli.

Reduced ER Ca\(^{2+}\) movement is associated with reduced cAMP levels

Our data show that although VX-809 causes only a small reduction in resting Ca\(^{2+}\), the dramatic reduction in ER Ca\(^{2+}\) release should affect Ca\(^{2+}\) signaling in the cells. Here, we focused on two adenylyl cyclases in renal cells that respond to Ca\(^{2+}\), AC3 and AC6 (31, 60). Western blotting confirmed that the PN cells contained both AC3 and AC6. Interestingly, VX-809 reduced the steady-state level of AC3 without producing any change in the AC6 level. Measurement of cAMP showed that VX-809 reduced cAMP levels by \(\sim 50\%\). Thus, the reduction in cAMP level resulting from VX-809 application is most likely the result of its reduction of AC3 levels. To deter-

**Figure 10. VX-809 has a small effect on apoptosis.** Apoptosis was measured using the EnzChek caspase-3 assay kit (Invitrogen, E13184). Cells were treated either with VX-809 or with DMSO at the indicated concentrations for 16 h or left untreated (control). Both treated and control cells were then harvested, lysed, and assayed as described in the manufacturer’s protocol. Reactions were carried out at room temperature, and fluorescence was measured in a fluorescence microplate reader (SpectraMax M3) with excitation at 496 nm and emission detection at 520 nm. Background fluorescence, determined for a no-enzyme control, was subtracted from each value. Ac-DEVD-CHO was used as an inhibitor control (inhib-cntrl) to confirm the correlation between signal detection and caspase-3-like protease activity. Camptothecin (Cam) was purchased from Selleckchem (catalog No. S1288). It is known to induce apoptosis (64) and was added as a positive control. Data were analyzed using one-way analysis of variance multiple comparisons. \(n = 4\)–5, *, \(p < 0.05\); ***, \(p < 0.0001\).

**Figure 11. Expression of ER stress markers GRP78 and ErO1 are unchanged, but GADD153 level is altered in Pkd1\(^{-/-}\) mice.** A and D, representative Western blot images of GRP78, ErO1, and GADD153 in lysates of kidney tissue from no cyst induced (ND), cyst induced with doxycycline (D), or cyst induced in pkd\(^{-/-}\) mice treated with VX-809 (D+VX809), 30 mg/kg body weight. B, C, and E, columns represent averages ± S.E. of GRP78, ErO1, and GADD153 expression. Data were analyzed by nonparametric t test. \(n = 4\) for each treated and control group. Black lines between lanes indicate that experiments are from the same gel but loaded in different lanes. **, \(p < 0.01\); ***, \(p < 0.001\). A and B are representative images extracted from the same gel.
The corrector VX-809 slows cyst growth in ADPKD

GADD expression in kidneys of Pkd1fl/fl;Pax8rtTA;TetO-cre mice.

Figure 12. GADD153 expression is reduced in kidneys of Pkd1fl/fl;Pax8rtTA;TetO-cre mice. Immunofluorescence images representing GADD153 expression (green) in DMSO (A) or VX-809 (B)–treated PN21 Pkd1fl/fl mouse. C, columns represent the % area of GADD153 immunopositive (green) cells in DMSO– or VX-809–treated PN21 Pkd1fl/fl mouse kidney sections. Data are expressed as mean ± S.E. of DMSO–treated (n = 4) and VX-809–treated (n = 4) mice. Statistical analysis was performed using a 2-tailed Student t test. Kidneys were fixed in 4% paraformaldehyde as described (25). Used here were GADD153 antibody (Sc-7351), goat anti-rabbit (A21429, Alexa Fluor 555, 1:1,000, Life Technologies), and 4′,6-diamidino-2-phenylindole (H-1200, Vector Laboratories, Burlingame, CA). Pictures were acquired with a Zeiss microscope equipped with a ×20 objective. Cells positive for GADD153 and the total number of cells were measured with ImageJ. The results are expressed as percentages. *, p < 0.05.

Conclusion

We have shown here that VX-809 has the surprising ability to restore renal cells in ADPKD to a noncyst-forming phenotype, including negating the ability of cAMP to sustain and stimulate cyst growth. A key result is that VX-809 reduced Hsp27, Hsp70, and Hsp90, which are known to be up-regulated in cancer cells to protect the proteins in abnormally dividing cells (61). Although the CFTR is a major driver of cyst growth, it is apparently not a major player in the normal kidney, because patients with CF do not have kidney disease. VX-809, in removing the cyst’s ability to grow in the presence of high levels of cAMP and inhibiting Ca2+ release by the ER, turns off or reduces two major signal transduction pathways that fuel cyst growth (see Fig. 13). Given that administration of VX-809 has already been shown to be safe, we conclude that it could offer a new approach for treating patients with ADPKD.

Methods

Cell culture and reagents

PN and PH cells, cultured as described previously (21, 22), were obtained from the Mouse Genetics and Cell Line Core of the Yale O’Brien Center. Forskolin (catalog No. 11018) was purchased from Sigma (SC23950); VX-809 (S1565) was purchased from Selleck Chemicals, Houston, TX. Ezrin (SC58758), adenylate cyclase 3 (SC588), PC2 (SC28331), Hsp70 (SC66048), anti-GADD153 antibody (SC7351), anti-Erk1 antibody (SC365526), and β-actin (SC47778) were purchased from Santa Cruz Biotechnology. Hsp90 (ADL-SPA-830F) was purchased from Enzo Life Sciences. AC6 (GTX47798) was purchased from GeneTex, Irvine, CA. Anti-GRP78 BiP antibody was purchased from Abcam (catalog No. ab21685).

Mouse strain and treatment

All animal use complied with the guiding principles of The Johns Hopkins University Institutional Animal Care and Use Committee, and the protocols for this work were approved by that Committee. Pkd1fl/fl;Pax8rtTA;TetO-cre mice on a C57BL/6 background (62) were provided by the Baltimore Polycystic Kidney Disease Center. Mice of both sexes were used in this study. Mice were generally fed a diet containing low fiber (5%), protein (20%), and fat (5–10%). The pelleted feed was supplied as regular, breeder-certified, irradiated, or autoclavable. Mice were supplied food and water free choice. Water was supplied using automatic waterers. Mouse rooms were maintained at 30–70% relative humidity and a temperature of 18–26 °C with at least 10 room air changes/h. The mice were housed in standard shoebox cages without filter tops. Cages as described above were usually changed once or twice a week. Mice were provided with bedding in the shoebox cages. Bedding could be paper, wood shavings, wood chips or corncob.

To induce cyst formation, mice were injected IP with doxycycline (Sigma, D9891) (4 μg of doxycycline/g body weight) on PND11, PND12, and PND13 to produce very rapid and aggressive cyst growth (19, 20). On PND21, the mice were euthanized. Three groups of animals were used: 1) animals injected with
doxycycline and with VX-809 (30 mg/kg); 2) animals injected with doxycycline and DMSO; 3) control animals not injected with doxycycline. Animal experiments were repeated 4–5 times to establish statistical significance.

**In vitro cystogenesis**

To induce differentiation, cells were kept at 37 °C for at least 6 days without γ-interferon. After 1 week, the cells were used for 3D culture or other experiments. Growth factor–reduced Matrigel (Corning, catalog No. 354230) was used, and cell dilutions were prepared so that there were 6000 cells in 25 μl of medium; 25 μl of cell preparation was mixed with 50 μl of Matrigel (see Ref. 26). Pictures were taken with a Zeiss Axio microscope. Cystic areas were analyzed with ImageJ (provided by National Institutes of Health).

**cAMP assay**

Confluent cells were treated with VX-809 (10 μM) or DMSO for 16 h before being harvested for assay. cAMP levels were measured with a direct cAMP enzyme immunoassay kit (Sigma, CA200) according to the manufacturer’s protocol. The results are expressed as pmol/ml. Statistical analysis was performed using a two-tailed Student’s t test.

**Fura-2 Ca²⁺ imaging assay**

On day 5 of cell culture, the cells were loaded with the cell-permeant acetoxymethyl (AM) ester of the calcium indicator Fura-2 AM at 37 °C for 90 min. Measurements were made on a Zeiss inverted microscope equipped with a Sutter Lambda 10-2 controller and filter wheel assembly. For ATP stimulation experiments, the cells were exposed to 100 μM ATP diluted in the imaging buffer. A Zeiss FluorArc mercury lamp was used to excite the cells at 340 and 380 nm, and the emission response was measured at 510 nm. Cell fluorescence was measured in response to excitation for 1000 ms at 340 nm and 200 ms at 380 nm once every 4 s. Image acquisition, image analysis, and filter wheel control were performed with IPLab software (see Ref. 26).

**Statistics**

All experiments were replicated until statistically significant at p < 0.05. All experiments were unblinded.
References


Acknowledgments—We thank William B. Guggino for reading the manuscript and Deborah McClellan for editorial assistance. We also extend special thanks to Cristina Cebotaru for work on the experiments.
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