High-throughput chemical screening identifies AG-490 as a stimulator of aquaporin 2 membrane expression and urine concentration

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Diabetes insipidus (DI) is characterized by a reduction in urine concentrating ability; the main symptoms of DI are polyuria with associated polydipsia, dehydration, and hypernatremia. Both central DI (due to loss of vasopressin [VP]) or nephrogenic DI (NDI) can be hereditary or acquired. In adults, acquired NDI is more common and it is most often due to chronic lithium use or hypercalcemia. In patients with hereditary NDI, symptoms usually appear soon after birth; the most severe complications are renal insufficiency and mental retardation. Hereditary NDI is caused by mutations in two genes, the V2 vasopressin receptor (V2R) and the aquaporin 2 (AQP2) water channel (11, 39). Ninety percent of hereditary NDI is caused by inactivating mutations in V2R, and the other 10% results from AQP2 mutations (13, 34). While central DI is usually manageable by administration of desmopressin (modified vasopressin) by nasal spray, for example, treating NDI patients is more problematic because of their resistance to vasopressin. They are frequently submitted to salt restriction, thiazide diuretics, and prostaglandin inhibitors (12, 22), but these therapies have only limited benefit.

VP-induced trafficking and membrane accumulation of the water channel AQP2 plays a critical role in maintaining water balance. Our understanding of this process at the cellular level has increased considerably over the past few years and has informed directed attempts to bypass defective VP signaling to increase urinary concentrating ability in DI. We now know, for example, that AQP2 recycles constitutively between cytosolic vesicles and the apical membrane, and the amount of AQP2 on the apical membrane at any given time is determined by a balance of exocytosis and endocytosis (7, 18, 24, 25, 27). We also know that phosphorylation of AQP2 is required to maintain this channel at the cell surface following its exocytotic insertion. AQP2 membrane accumulation then allows water to permeate through the plasma membrane, resulting in urine concentration.

Recent studies from our group and others have suggested that bypassing the defective V2R (which accounts for 90% of hereditary NDI cases) to activate AQP2 membrane accumulation in kidney principal cells is a feasible and promising therapeutic strategy. Some compounds, for example a phosphodiesterase type 5 (PDE5) inhibitor (sildenafil) (5), a phosphodiesterase type 4 (PDE4) inhibitor (rolipram) (35), statins (simvastatin, fluvastatin, and lovastatin) (21, 32), calcitonin (4), and selective agonists for E-prostanoid receptors (20, 30), successfully increased AQP2 plasma membrane accumulation. However, it remains uncertain whether these compounds can be translated into humans as a therapeutic strategy. For example, use of rolipram did not help patients with hereditary X-linked NDI (1). Therefore, additional approaches and new drugs are still needed to bypass the defective V2R/VP signaling pathway in both acquired and hereditary DI. The compounds discussed above were identified rationally on the basis of our understanding of known AQP2 signaling/trafficking pathways. However, this approach is limited and has tended to focus on mechanisms related to the cAMP signaling pathway. In contrast, unbiased screening

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of chemical libraries using appropriate assays may provide unexpected novel therapeutic strategies.

In the present report we have, therefore, established and validated a nonbiased high-throughput chemical screening method which, when coupled to additional downstream assays, was designed to identify compounds that might modulate principal cell water permeability by affecting AQP2 membrane accumulation. Initial chemical screening was performed by adapting our fluorescence-based exocytosis assay (29) to a high-throughput format. The purpose of this screening was to quickly identify compounds that modulate exocytosis in renal epithelial cells. From the initial candidates we next identified those compounds that regulate specifically AQP2 trafficking first in cultured renal epithelial cells, then in kidney slices in vitro, and finally using in vivo whole animal studies. 1

MATERIALS AND METHODS

High-throughput screening assay. This screening assay was developed from the exocytosis assay that was described in detail in a previous study (29). LLC-PK1 cells expressing both AQP2 and soluble secreted yellow fluorescent protein (ssYFP) were seeded at a density of 2 × 10^3 cells/well on 384-well microplates using a Multidrop Combi reagent dispenser (Thermo Scientific, Waltham, MA) and then cultured until confluence (3 days). Cells were washed

Table 1. Candidate AQP2 exocytosis enhancer compounds

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Final Concentration, μM</th>
<th>NEx ± SD</th>
<th>Description</th>
<th>IF Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurotransmitter modulators</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Salsoline</td>
<td>50</td>
<td>1.37 ± 0.09</td>
<td>Dopamine-related compound</td>
<td>NA</td>
</tr>
<tr>
<td>2) (S)-Lisuride</td>
<td>10</td>
<td>1.45 ± 0.03</td>
<td>MAO-A inhibitor, serotonin</td>
<td>NA</td>
</tr>
<tr>
<td>3) Ergonovine</td>
<td>10</td>
<td>1.45 ± 0.03</td>
<td>Dopamine/serotonin agonist</td>
<td>NA</td>
</tr>
<tr>
<td>4) Benzerazide hydrochloride</td>
<td>7.77</td>
<td>1.54 ± 0.01</td>
<td>AAAD decarboxylase inhibitor, antiparkinsonian</td>
<td>NA</td>
</tr>
<tr>
<td>5) Agroclavine</td>
<td>10</td>
<td>1.59 ± 0.02</td>
<td>D2 agonist, D1 antagonist, serotonin agonist</td>
<td>NA</td>
</tr>
<tr>
<td>6) Fluphenazine</td>
<td>10</td>
<td>1.64 ± 0.03</td>
<td>Adrenergic or serotonin receptor</td>
<td>CT</td>
</tr>
<tr>
<td>7) Trilthoperazine</td>
<td>10</td>
<td>1.65 ± 0.1</td>
<td>Calmodulin inhibitor, antiadrenergic/dopaminergic</td>
<td>AK</td>
</tr>
<tr>
<td>8) Menadione</td>
<td>50</td>
<td>1.78 ± 0.08</td>
<td>DOPA decarboxylase inhibitor</td>
<td>AF</td>
</tr>
<tr>
<td>9) Dobutamine hydrochloride</td>
<td>50</td>
<td>1.85 ± 0.04</td>
<td>A-β-2 agonist</td>
<td>NC</td>
</tr>
<tr>
<td>Lipid receptor ligands</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10) U-46619</td>
<td>5</td>
<td>1.41 ± 0.18</td>
<td>Thromboxane TP receptor agonist</td>
<td>NC</td>
</tr>
<tr>
<td>11) Prostaglandin E2</td>
<td>5</td>
<td>1.65 ± 0.12</td>
<td>Prostaglandin EP receptor agonist</td>
<td>AK</td>
</tr>
<tr>
<td>Kinase inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12) Emnidin</td>
<td>50</td>
<td>1.46 ± 0.12</td>
<td>Tyrosine kinase inhibitor, CK2 inhibitor, natural</td>
<td>AF</td>
</tr>
<tr>
<td>13) AG-490</td>
<td>85</td>
<td>1.46 ± 0.06</td>
<td>JAK-2 kinase and EGFR inhibitor</td>
<td>PH</td>
</tr>
<tr>
<td>14) Y-27632</td>
<td>100</td>
<td>1.46 ± 0.2</td>
<td>ROCK-1 kinase inhibitor</td>
<td>AK</td>
</tr>
<tr>
<td>15) Tyrophostin AG-825</td>
<td>63</td>
<td>1.55 ± 0.26</td>
<td>HER-1,2 tyrosine kinase inhibitor</td>
<td>NC</td>
</tr>
<tr>
<td>16) RO51-8220</td>
<td>54.5</td>
<td>1.66 ± 0.11</td>
<td>PKC inhibitor</td>
<td>AF</td>
</tr>
<tr>
<td>17) AG-213</td>
<td>115</td>
<td>1.97 ± 0.01</td>
<td>EGFR tyrosine kinase inhibitor</td>
<td>NC</td>
</tr>
<tr>
<td>18) GF-109203X</td>
<td>60.5</td>
<td>2.96 ± 0.12</td>
<td>PKC inhibitor</td>
<td>AF</td>
</tr>
<tr>
<td>Other inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19) β-Lapachone</td>
<td>105</td>
<td>1.46 ± 0.03</td>
<td>Topoisomerase I inhibitor, apoptosis inducer</td>
<td>PH</td>
</tr>
<tr>
<td>20) NSC-95397</td>
<td>80.5</td>
<td>1.75 ± 0.28</td>
<td>CDC25 phosphatase inhibitor</td>
<td>CT</td>
</tr>
<tr>
<td>21) Dipryridamole</td>
<td>3.96</td>
<td>2.09 ± 0.09</td>
<td>Phosphodiesterase inhibitor</td>
<td>AK</td>
</tr>
<tr>
<td>Cytotoxic/apoptosis-inducing compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22) 1,4-Naphthoquinone</td>
<td>50</td>
<td>1.36 ± 0.06</td>
<td>Antibacterial, anticancer</td>
<td>NA</td>
</tr>
<tr>
<td>23) Dalbergione</td>
<td>50</td>
<td>1.45 ± 0.09</td>
<td>Severe skin irritant, anti-cancer</td>
<td>NA</td>
</tr>
<tr>
<td>24) Quinalizarin</td>
<td>50</td>
<td>1.49 ± 0.04</td>
<td>Cytotoxic dye, inhibitor of CK2, induces apoptosis</td>
<td>NA</td>
</tr>
<tr>
<td>25) Shikonin</td>
<td>86.5</td>
<td>1.55 ± 0.07</td>
<td>Apoptosis inducer, p53-dependent</td>
<td>AF</td>
</tr>
<tr>
<td>26) Pyromycin</td>
<td>50</td>
<td>1.58 ± 0.03</td>
<td>Anthracyle antibiotic, inhibits RNA synthesis</td>
<td>NA</td>
</tr>
<tr>
<td>27) Aklavine hydrochloride</td>
<td>50</td>
<td>1.60 ± 0.07</td>
<td>Cytotoxic activity against multidrug-resistant cells</td>
<td>NA</td>
</tr>
<tr>
<td>28) Antimycin a</td>
<td>50</td>
<td>1.73 ± 0.05</td>
<td>Induces apoptosis, inhibits mitochondria function</td>
<td>NC</td>
</tr>
<tr>
<td>29) HA14-1</td>
<td>61</td>
<td>1.76 ± 0.06</td>
<td>Bel-2 ligand induces apoptosis</td>
<td>PH</td>
</tr>
<tr>
<td>30) Doxorubicin hydrochloride</td>
<td>3.68</td>
<td>1.97 ± 0.06</td>
<td>DNA-intercalating agent</td>
<td>AF</td>
</tr>
<tr>
<td>Other natural products</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31) Brazilin</td>
<td>50</td>
<td>1.48 ± 0.05</td>
<td>Haematoxylin-like may protect from oxidative stress</td>
<td>NA</td>
</tr>
<tr>
<td>32) Bazzilein</td>
<td>50</td>
<td>1.50 ± 0.01</td>
<td>Anti-inflammatory, lowers cytokines TNF-α, IL-6</td>
<td>NA</td>
</tr>
<tr>
<td>33) Epigallocatechin 3,5-digallate</td>
<td>50</td>
<td>1.54 ± 0.01</td>
<td>Tea extract polyphenol, inhibits insulin secretion</td>
<td>PH</td>
</tr>
<tr>
<td>34) Purpurein</td>
<td>50</td>
<td>1.67 ± 0.06</td>
<td>Red/yellow dye, unknown</td>
<td>AF</td>
</tr>
<tr>
<td>35) 7-Hydroxy-2-methoxyisoflavone</td>
<td>50</td>
<td>1.88 ± 0.06</td>
<td>Isoflavonoid, unknown</td>
<td>NA</td>
</tr>
<tr>
<td>36) 10Dimenthyl bicycle [7:2:0] undecane</td>
<td>50</td>
<td>2.68 ± 0.14</td>
<td>Unknown</td>
<td>NA</td>
</tr>
</tbody>
</table>

Thirty-six candidate enhancers of aquaporin 2 (AQP2) exocytosis were identified from primary screening of 3,464 compounds with a soluble secreted yellow fluorescent protein (ssYFP) exocytosis assay. These candidates were evaluated by secondary screening with immunofluorescence (IF). NEx, normalized exocytosis score; NA, not available commercially; CT, cytotoxic; AK, already known as enhancer of AQP2 membrane accumulation (compound 7, 11, 24, 21); AF, autofluorescence; NC, no change of localization of AQP2; PH, positive hit with immunofluorescence (compound 13, 19, 32). AK and PH compounds are highlighted in bold. AAAD, aromatic L-amino acid decarboxylase; EGFR, epidermal growth factor receptor; ROCK-1, Rho-associated protein kinase 1.
with Hank’s balanced salt solution HBSS (containing 20 mM HEPES and 2 g/l glucose) using a BioTek ELx 405 microplate washer (BioTek US, Winooski, VT) and then incubated in HBSS for 1 h before chemical delivery. Compounds were from the Prestwick Chemical Library of agency-approved (including the FDA), off-patent drugs (Prestwick Chemical, Illkirch, France), Spectrum Collection (Microsource Discovery Systems, Gaylordsville, CT), and the supplemented ICCB (Harvard Institute of Chemistry and Cell Biology) known bioactives collection libraries. Compounds were delivered to each well using the 100 nl 384-pin head attachment of a CyBio-Well Vario robotic pipettor (CyBio US, Woburn, MA). After pinning, the cells were incubated for 30 min at 37°C, then the extracellular medium was removed and delivered to 384 black low-volume non-binding surface microplates (Corning, Tewksbury, MA). Plates were spun at 100 g for 1 min to collect medium at the bottom of the plates, and fluorescence was read on a SafireII multiwavelength fluorimeter (Tecan Systems, San Jose, CA). The exocytosis (Ex) score was defined as the secreted ssYFP fluorescence divided by the sum of the secreted ssYFP fluorescence and the ssYFP fluorescence remaining in the lysates. Ex scores were then normalized by the DMSO (or HBSS for those compounds dissolved in aqueous solutions) to give the normalized exocytosis score (NEx) score. The NEx scores of the triplicate wells were averaged. Z-scores are defined as follows: \[ Z = \frac{[NEx - \mu_o]/\sigma_o}{\sigma} \], where \( \mu_o \) and \( \sigma_o \) are the DMSO NEx population mean and standard deviation, respectively. Positive hits listed in Table 1 were chemicals for which the absolute value of the Z-score was greater than 2.5.

Immunofluorescence staining. Immunofluorescence with LLC-PK1 cells and Madin Darby canine kidney (MDCK) cells stably expressing AQP2 (17, 42) was performed as previously described (40, 41). LLC-PK1 cells and MDCK cells were cultured on glass coverslips and polyester filters (Corning), respectively. The MDCK cells were treated with 50 \( \mu \)M indomethacin overnight as previously described (10, 41). After 2 h of serum starvation, the cells were incubated with compounds for 30 min and fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100-PBS, and blocked in 1% BSA-PBS. Goat anti-AQP2 C-17 antibody (0.5 \( \mu \)g/ml, Santa Cruz Biotechnology, Dallas, TX) and donkey anti-goat IgG conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA) were used as a primary antibody and a secondary antibody, respectively. Images were acquired with a Zeiss Radiance 2000 confocal laser-scanning microscope or a Nikon 80i microscope. AG-490 was obtained from Selleck Chemicals (Houston, TX). β-Lapachone and epigallocatechin gallate

Fig. 1. Flow chart showing the screening and testing procedures used in this study. The boxes show the number of compounds that remained after each screening step. From an initial pool of 3,464, only one compound remained that increased urine concentration in rats while activating membrane accumulation of aquaporin 2 (AQP2) in vivo. ssYFP, soluble secreted yellow fluorescent protein.

Fig. 2. AQP2 accumulates on the plasma membrane after treatment of LLC-PK1 cells with candidate compounds. A: representative AQP2 staining in LLC-PK1 cells stably expressing AQP2 after treatment with AG-490 (40 \( \mu \)M), β-lapachone (β-lap, 50 \( \mu \)M), epigallocatechin gallate (EGCG, 50 \( \mu \)M), HA14-1 (60 \( \mu \)M), and lysine-vasopressin/forskolin (VP/FK, 10 nM/10 \( \mu \)M) for 30 min. B: quantification of membrane AQP2 accumulation in LLC-PK1 cells. Mean fluorescence intensity in the plasma membrane was divided by mean fluorescence intensity in the cytosol. Data are shown as means ± SE; \( n = 4 \); NS, not significant. * \( P < 0.05 \) vs. control. The images presented here were all subjected to enhancement and sharpening of the entire field using the levels command and/or the high-pass filter in Adobe Photoshop. Images for quantification were all used without any enhancement.
EGCG were obtained from Enzo Life Sciences (Farmingdale, NY) and Sigma-Aldrich (St. Louis, MO), respectively.

Animal experiments. Animal experiments were approved by the Massachusetts General Hospital Institutional Committee on Research Animal Care in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For in vitro kidney slice experiments, kidney slices were prepared as described previously (4, 5). Briefly, adult Sprague-Dawley rats were anesthetized using isoflurane. Kidneys were harvested, and slices of ~0.5 mm were cut using a Stadie-Riggs microtome. All of the sliced kidneys were incubated at 37°C for 15 min in equilibrated HBSS (pH 7.4, with 5% CO2). After equilibration, the slices were incubated in HBSS containing chemicals (100 μM arginine vasopressin and 10 μM forskolin, 40 μM AG-490, 50 μM β-lapachone, or 0.1% DMSO as control) for 30 min. After incubation, all of the samples were immersed in 4% paraformaldehyde-lysine-periodate (PLP) fixative.

Fig. 3. Confocal immunofluorescence localization of AQP2 in Madin-Darby canine kidney (MDCK) cells stably expressing AQP2. The cells were cultured on filters. Top: apical slice of cells (i.e., the apical plasma membrane domain). Middle: middle portion of the cells. Bottom: xz-stack slice. AQP2 was located in the cytosol under basal conditions. After 30 min of incubation with VP/FK, β-lapachone, and AG-490, AQP2 accumulated at the apical pole (compare top panels with control). The concentrations of compounds were as same as in Fig. 2A with LLC-PK1 cells. The images presented here were all subjected to enhancement and sharpening of the entire field using the levels command and/or the high-pass filter in Adobe Photoshop.

Fig. 4. AQP2 accumulates on the apical plasma membrane in rat kidney slices after treatment with candidate compounds in vitro. Kidney slices from a Sprague-Dawley rat were incubated for 30 min with VP/FK (10 nM/10 μM), AG-490 (40 μM), or β-lapachone (50 μM). A: in inner medulla, AQP2 accumulated on apical membranes after VP/FK or AG-490 treatment. β-Lapachone had no detectable effect. B: in cortical collecting duct, AQP2 accumulated on apical membranes of principal cells (arrows) after VP/FK, AG-490, or β-lapachone treatment. Scale bars, 20 μm. C and D: quantitative analysis of AQP2 in inner medulla (C) and cortex (D). Fluorescence intensity of the apical area was divided by fluorescence of the whole cell area. Data are shown as means ± SE; n = 3. *P < 0.05 vs. control.
metabolic cage studies, each adult Brattleboro rat (from the Rat Resource and Research Center, Columbia, MO) was placed individually in a metabolic cage. Before the experiment, rats were allowed to acclimate to metabolic cages for 5 days. AG-490, β-lapachone, or the same amount of control solution (50% DMSO and 50% ethanol) was injected into rats intraperitoneally. The initial dose of each drug was determined by reference to previous studies (9, 19, 26, 38). We used different doses of chemicals and finally we chose a dose that had no significant side effects on animal behaviors such as food intake, grooming behavior, and general appearance of well-being. The metabolic cage study was repeated twice with a 10 days break period between treatments. Data from each rat were, therefore, determined by the average of two independent experiments. For blood sampling and kidney fixation, rats were anesthetized with isoflurane 2 h after drug injection. Blood was collected from the abdominal vena cava, and kidneys were immediately perfused through the left ventricle with PBS then with the PLP fixative. Cryosections were prepared as described previously (4). These specimens were stained with anti-AQP2 antibody (see above). Serum and urine were analyzed at the Massachusetts General Hospital Clinical Pathology Laboratory facility. Urine osmolality was analyzed with a Vapor Pressure Osmometer (Wescor, Logan, UT).

Quantification of AQP2 plasma membrane accumulation. The fluorescence of AQP2 labeling at the plasma membrane was quantified using Volocity software (PerkinElmer, Santa Clara, CA). For the quantification, LLC-PK1 cells were costained with wheat germ agglutinin (WGA 2 μg/ml; Lectin Kit, Vector Labs, Burlingame, CA) to clearly delineate the plasma and nuclear membranes. The mean fluorescence intensity of at least 30 regions of plasma membrane and cytosol from each of 5 different images from each sample taken at ×40 magnification were analyzed. The plasma membrane fluorescence was divided by the mean cytosolic value of each sample to normalize the values among different experiments. For quantification in rat kidney tissue, the fluorescence level of apical AQP2 and total cellular AQP2 was determined by measuring the intensity levels of a region of interest (ROI) encompassing only the apical membrane, and the whole cell area including the apical membrane domain, respectively. At least four tubules and three cells in each tubule were measured in each of three different areas of kidney (cortex, outer medulla, and inner medulla). Each experiment was repeated at least three times.

cAMP and cGMP assays. Total cellular cAMP and cGMP levels were measured with the BioTrak EIA system (GE Healthcare Life Sciences, Piscataway, NJ) as described in detail previously (4, 5). Cells treated with VP (10 nM) or sodium nitroprusside (NTP; 1 mM) for 10 min were used as positive controls. Each assay was performed in triplicate and repeated three times independently.

Statistical analysis. Statistical analyses were performed using the unpaired t-test. P values <0.05 were considered statistically significant.

RESULTS

High-throughput screening assay. The total amount of ssYFP secreted into the extracellular medium was measured in LLC-PK1 cells expressing both ssYFP and AQP2 (LLC-AQP2-ssYFP cells) (29) in the presence of 3,464 bioactive molecules. We identified 36 candidate exocytosis enhancers that increased ssYFP medium fluorescence (Table 1). In our unbiased study, we also detected some compounds already known to regulate AQP2 trafficking: prostaglandin E2 (20, 30), dipyridamole (5, 35, 36), and Y-27632 (37). These results, which serve as positive controls, indicate that our screening system worked effectively. However, one of the compounds picked up as an exocytosis enhancer was trifluoperazine, which has been reported to inhibit VP-dependent water permeability and AQP2 trafficking (8). This indicates the need to perform careful confirmatory follow-up experiments after initial high-throughput screening of chemical libraries. Such studies are reported below, and a flow chart of the entire screening and testing procedure is illustrated in Fig. 1. The chart shows the number of compounds surviving each step of the procedure.

Testing candidate compounds on cultured cells and kidney tissue slices in vitro. The ssYFP exocytosis assay indicated whether a given compound could stimulate or inhibit the accumulation of ssYFP in the culture medium, but it did not prove directly its effect on membrane accumulation of AQP2. “False positives” can occur if a compound is autofluorescent or causes cell death or lysis. Therefore, we performed immunofluorescence studies to determine AQP2 localization in cultured cells exposed to candidate enhancer compounds. We excluded 4 drugs (prostaglandin E2, dipyridamole, trifluoperazine, and Y-27632) that were already known to affect AQP2 trafficking, and 13 drugs were not commercially available. Finally, we tested 19 enhancer candidates by immunofluorescence with AQP2 expressing LLC-PK1 cells. Treatment with 50 μM β-lapachone, 40 μM AG-490, and 60 μM HA14-1 caused significant plasma membrane accumulation of AQP2 (Fig. 2A). Epigallocatechin gallate (EGCG, 50 μM) caused some apparent membrane accumulation by visual inspection, but the effect did not reach statistical significance upon quantification (Fig. 2B), even after increasing the concentration of EGCG to 200 μM (data not shown).

Seven compounds initially identified (menadione, emodin, RO31-8220, GF-109203X, shikonin, doxorubicin hydrochloride, and purpurin) were autofluorescent and had no effect on AQP2 trafficking (Table 1). Two compounds (fluphenazine and NSC-95397) were cytotoxic (Table 1). Six others (dobutamine hydrochloride, U-46619, tyrphostin AG-825, AG-213, quinazolin, and antimycin A) had no significant effect on the localization of AQP2 in LLC-PK1 cells (Table 1).

We further examined the effect of candidate compounds using MDCK cells stably expressing AQP2 to determine the generality of the effect of enhancer compounds on AQP2 trafficking. Both β-lapachone and AG-490 caused a marked...
accumulation of AQP2 on the apical membrane that mimicked the effect of VP (Fig. 3). However, the effect of EGCG and HA14-1 on AQP2 membrane accumulation was not significant in MDCK cells (data not shown).

We then tested these compounds on rat kidney slices in vitro. After AG-490 treatment, AQP2 accumulated on the apical membrane of principal cells in both inner medulla (Fig. 4, A and C) and cortex (Fig. 4, B and D). β-Lapachone treatment induced AQP2 accumulation on the apical membrane in the cortex only, and not in the medulla. We also applied EGCG and HA14-1 to kidney tissue slices, but as in MDCK cells, these compounds had no significant effect on AQP2 localization (data not shown).

Effect of compounds on urinary concentration in VP-deficient Brattleboro rats. To evaluate the effect of compounds on urine volume and osmolality in vivo, VP-deficient Brattleboro rats were housed individually in metabolic cages. These rats, which model central DI, have been used in the past to identify drugs that can increase urine concentration in the complete absence of the antidiuretic hormone VP. Rats were injected intraperitoneally with AG-490 (35 mg/kg), β-lapachone (17 mg/kg), or control solution. After AG-490 injection, urine volume was significantly decreased and urine osmolality was significantly increased within 2 h after injection (Fig. 5, A and B). However, this effect of AG-490 did not continue after 6 h, and urine volume and osmolality after 8 h was almost the same as in control rats (Fig. 5). Despite a potent effect on AQP2 trafficking in all of the in vitro systems we

Table 2. Twenty-four hour metabolic cage study data

<table>
<thead>
<tr>
<th></th>
<th>Time 0</th>
<th>24 h</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Food intake, g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17.4 ± 0.4</td>
<td>16.7 ± 0.7</td>
<td>0.96 ± 0.04</td>
</tr>
<tr>
<td>AG-490</td>
<td>14.5 ± 2.1</td>
<td>14.7 ± 1.8</td>
<td>1.02 ± 0.12</td>
</tr>
<tr>
<td>β-Lap</td>
<td>14.3 ± 3.5</td>
<td>10.9 ± 2.6</td>
<td>0.75 ± 0.18</td>
</tr>
<tr>
<td><strong>Feces, g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.6 ± 1.2</td>
<td>10.0 ± 2.5</td>
<td>1.17 ± 0.29</td>
</tr>
<tr>
<td>AG-490</td>
<td>7.8 ± 2.2</td>
<td>7.4 ± 1.9</td>
<td>0.94 ± 0.24</td>
</tr>
<tr>
<td>β-Lap</td>
<td>6.4 ± 5.4</td>
<td>6.4 ± 2.3</td>
<td>1.00 ± 0.36</td>
</tr>
<tr>
<td><strong>Body wt, g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>468 ± 20</td>
<td>463 ± 19</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td>AG-490</td>
<td>475 ± 24</td>
<td>472 ± 25</td>
<td>0.99 ± 0.05</td>
</tr>
<tr>
<td>β-Lap</td>
<td>494 ± 29</td>
<td>485 ± 29</td>
<td>0.98 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3. Experiments were repeated twice, and an average for each rat was obtained. Ratio was defined as after-treatment (24 h) values divided by before-treatment (time 0) values. There were no significant differences between ratio values of treated animals compared with control untreated animals. β-Lap, β-lapachone.
examined, β-lapachone failed to show any significant effect on urine volume and osmolality in the time period tested (Fig. 5).

We next determined the subcellular distribution of AQP2 after in vivo drug treatment. Kidneys from each group of treated Brattleboro rats were harvested 2 h after injection and were immunostained for AQP2 (Fig. 6A). There was a significant accumulation of AQP2 at the apical membrane in the cortex of AG-490-treated rats, in contrast to the more diffuse distribution in vehicle-treated rats (Fig. 6A and B). However, there was no significant enhancement of AQP2 apical localization in principal cells in vivo, in contrast to the effect on kidney tissue slices in vitro. β-Lapachone had no detectable effect on AQP2 localization in any area of the kidney in vivo. These observations suggest that there are potential differences in bioavailability and drug delivery to target cells in vivo and in vitro.

Potential side effects of these chemicals were also examined. After the treatment, the rats looked normal and showed normal behaviors. There were no significant differences in food intake and body weight change after normalization compared with the control group (Table 2). There was no significant difference in blood chemistries, including kidney and liver function tests (Table 3). Finally, there was no significant difference in creatinine clearance (Table 3), suggesting adequate renal function in these animals. Therefore, the reduction in urine volume seen in AG-490-treated animals was unlikely to have resulted from a reduction of the glomerular filtration rate.

**AG-490 and β-lapachone increase exocytosis only when AQP2 is expressed.** To determine the specificity of AG-490 on AQP2 exocytosis, we performed additional ssYFP exocytosis assays (Fig. 7). AG-490 increased exocytosis in LLC-AQP2-ssYFP cells, but it did not increase exocytosis in LLC-PK1 cells that do not express AQP2. This indicates that the increase in exocytosis induced by AG-490 requires expression of AQP2. These data are similar to those seen after VP treatment (Fig. 7) which, as we previously showed, increases exocytosis only in cells that express AQP2 (29, 41). Interestingly, β-lapachone, a compound that increased AQP2 membrane accumulation in cell cultures and in tissue slices (see above), also required AQP2 expression to stimulate ssYFP exocytosis. This again indicates that its apparent lack of effect in vivo could result from a bioavailability problem.

**AG-490 does not increase cAMP or cGMP levels.** The significant effect of AG-490 on AQP2 trafficking prompted us to look at the effect of AG-490 on cAMP and cGMP levels to determine whether its effect on AQP2 trafficking might occur via the canonical cAMP/PKA pathway or the alternative cGMP pathway. As shown in Fig. 8, AG-490 did not increase cAMP or cGMP levels, whereas 1 mM sodium nitroprusside (NTP) had a highly significant effect (*P* < 0.05 vs. control). Similarly, AG-490 did not increase cGMP levels, whereas 1 mM sodium nitroprusside (NTP) had a highly significant effect (*n* = 3). Data are expressed in femtomoles of cAMP or cGMP generated per 10⁶ cells and are shown as means ± SE. *P* < 0.05 vs. control for VP and NTP effects.

### Table 3. Serum biochemistries show no significant differences after treatment

<table>
<thead>
<tr>
<th></th>
<th>BUN (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>TP (g/dl)</th>
<th>Glucose (mg/dl)</th>
<th>ALT (U/l)</th>
<th>ALP (U/l)</th>
<th>CCr (mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.5 ± 2.7</td>
<td>0.3 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>217 ± 57</td>
<td>39 ± 7</td>
<td>277 ± 19</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>AG-490</td>
<td>15.4 ± 5.3</td>
<td>0.3 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>187 ± 7</td>
<td>32 ± 7</td>
<td>239 ± 51</td>
<td>3.5 ± 1.8</td>
</tr>
<tr>
<td>β-Lapachone</td>
<td>15.9 ± 0.8</td>
<td>0.3 ± 0.0</td>
<td>5.2 ± 0.3</td>
<td>187 ± 45</td>
<td>42 ± 8</td>
<td>293 ± 51</td>
<td>4.3 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SD; *n* = 3. BUN, blood urea nitrogen (mg/dl); creatinine (mg/dl); TP, total protein (g/dl); glucose (mg/dl); ALT, alanine aminotransferase (U/l); ALP, alkaline phosphatase (U/l); CCr, creatine clearance (mg/min). CCr was obtained from 4 h urine collection after injection of compounds.
pathway that we have previously described (3, 5). As shown in
Fig. 8, AG-490 treatment with LLC-PK1 cells did not induce a
measurable elevation of cAMP or cGMP levels, in contrast to
the expected and strong effect of VP and NTP, respectively.

DISCUSSION

We describe here an unbiased screening procedure to iden-
tify novel chemicals that stimulate VP-independent AQP2
membrane trafficking. We interrogated selected libraries, includ-
ing off-patent, FDA-approved drugs and known bioactive com-
ounds, using a high-throughput modification of our ssYFP exo-
cytosis assay (29). By following the initial screening with in
vitro and in vivo studies, we identified AG-490 as a stimulator
of AQP2 membrane accumulation both in cell culture systems
and in principal cells in situ. AG-490 is marketed as a JAK-2
kinase and an EGFR receptor (EGFR) inhibitor. Importantly,
AG-490-treated, VP-deficient Brattleboro rats showed a signif-
icant and acute increase in urine osmolality and a significant
decrease in urine volume. There was no significant difference
in creatinine clearance between AG-490 treatment and controls
groups, indicating that a reduction in glomerular filtration rate
was not a major contributor to the decreased urine output. In vivo,
an increased membrane accumulation of AQP2 was detectable only in
the cortical collecting duct but not in medullary principal cells. This
effect still resulted in a significant increase in urine concentration,
because 60–70% of water reabsorption occurs in the cortical region
of the collecting duct under antidiuretic conditions (33).

Our initial screening also detected some known stimulators of
AQP2 trafficking (see Table 1), validating the methodology. A
recent study using a different screening approach identified inhib-
itors of AQP2 membrane accumulation (2). There, too, the ap-
proach was validated by the unbiased identification of a blocker of
V-ATPase (proton pumping ATPase) activity, which we had previ-
ously shown to prevent AQP2 trafficking (15). However,
some of the compounds identified in our original screen proved to
be false positives. Some were autofluorescent, and some were
 cytotoxic, resulting in cell damage and nonexocytotic release of
ssYFP. Furthermore, some compounds may have increased me-
dium ssYFP via exocytosis of vesicles that do not also contain
AQP2. Others, including β-lapachone, seemed promising in vitro
but had no detectable effect in vivo. This suggests a potential
problem with bioavailability that needs to be addressed in the
future. Importantly, however, neither AG-490 nor β-lapachone
increased exocytosis in cultured cells that do not express AQP2,
indicating that their effect depends on the presence of AQP2.
Indeed, we have previously reported that VP increases exocytosis
only in cells that express AQP2 (29), suggesting a relationship
between AQP2 and the exocytotic machinery activated by VP.
This result was repeated here (see Fig. 7). Our recent report
showed that this is correlated with VP-induced actin depolymer-
ization, which occurs only in AQP2-expressing cells (41). Previ-
ous studies have also revealed a close relationship between AQP2
and the actin cytoskeleton (23, 28, 37).

It is now appreciated that VP-independent AQP2 trafficking
can be modulated by various intracellular pathways that in-
clude cGMP (5), MAP kinases (16), calcium-activated path-
ways (8), prostaglandins (20, 30), and the actin cytoskeleton
(21, 32). Further studies will be required to clarify the precise
mechanism by which AG-490 bypasses the V2R signaling
pathway, but it is interesting that previous reports showed that

epidermal growth factor (EGF) inhibits the VP effect and
induces diuresis in vivo (6, 14, 31). A logical extension of this
result is that the reverse effect, i.e., an increase in urinary
concentration, might occur when the EGFR is inhibited by
AG-490. Whether the activity of this drug on the JAK-2
pathway or on EGFR signaling is involved in the mechanism
by which AQP2 membrane accumulation is stimulated remains
to be determined. However, our data do rule out an involve-
ment of cAMP elevation—the canonical pathway of VP sig-
aling—as well as cGMP signaling, which we had previously
described as an alternative pathway stimulating AQP2 mem-
brane accumulation (3, 5). AG-490 had no detectable effect on
the intracellular levels of these cyclic nucleotides.

In conclusion, we developed and applied a systematic
screening procedure that identified AG-490 as a compound that
stimulates AQP2 exocytosis, induces AQP2 membrane accu-
lation, and stimulates urine concentration in a VP-indepen-
dent manner. High-throughput chemical screening with our cell-
based assay followed by in vitro and in vivo testing has, therefore,
been effective in identifying unsuspected strategies for the poten-
tial treatment of water balance disorders, and in identifying novel
signaling pathways for the regulation of intracellular AQP2 traf-
ficking that can be pursued in future studies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

design of research; N.N., P.N., R.B., A.V.N., S.S., N.P., H.A.J.L., and D.B.
interpreted results of experiments; N.N., A.V.N., N.P., and D.B. prepared figures;
N.N. and D.B. drafted manuscript; N.N., P.N., R.B., A.V.N., S.S., E.U., H.A.J.L.,
and D.B. edited and revised manuscript; N.N., P.N., R.B., A.V.N., S.S., E.U., N.P.,
H.A.J.L., and D.B. approved final version of manuscript.

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