Structure-activity relationship studies of four novel 4-aminopyridine K⁺ channel blockers

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4-Aminopyridine (4AP) is a specific blocker of voltage-gated potassium channels (Kᵥ family) clinically approved for the symptomatic treatment of patients with multiple sclerosis (MS). It has recently been shown that [¹⁸F]3F₄AP, a radiofluorinated analog of 4AP, also binds to Kᵥ channels and can be used as a PET tracer for the detection of demyelinated lesions in rodent models of MS. Here, we investigate four novel 4AP derivatives containing methyl (-CH₃), methoxy (-OCH₃) as well as trifluoromethyl (-CF₃) in the 2 and 3 position as potential candidates for PET imaging and/or therapy. We characterized the physicochemical properties of these compounds (basicity and lipophilicity) and analyzed their ability to block Shaker K⁺ channel under different voltage and pH conditions. Our results demonstrate that three of the four derivatives are able to block voltage-gated potassium channels. Specifically, 3-methyl-4-aminopyridine (3Me4AP) was found to be approximately 7-fold more potent than 4AP and 3F₄AP; 3-methoxy- and 3-trifluoromethyl-4-aminopyridine (3MeO₄AP and 3CF₃₄AP) were found to be about 3- to 4-fold less potent than 4AP; and 2-trifluoromethyl-4-AP (2CF₃₄AP) was found to be about 60-fold less active. These results suggest that these novel derivatives are potential candidates for therapy and imaging.

In normally myelinated neurons, voltage-gated potassium (K⁺) channels Kᵥ1.1 and Kᵥ1.2 are clustered near the nodes of Ranvier beneath the myelin sheath. Upon demyelination, these channels become exposed, migrate through the demyelinated segment and concomitantly increase in expression several fold. This aberrant redistribution of K⁺ channels impairs conduction of action potentials, which leads to neurological deficits. 4-Aminopyridine (4AP) is a selective blocker of Kᵥ channels used clinically to improve neurological conduction in people with multiple sclerosis (MS) and other demyelinating diseases. Mechanistically, 4AP blocks the exposed K⁺ channels and therefore enhances conduction. Recently, it has been shown that the fluorinated derivative 3-fluoro-4-aminopyridine (3F₄AP) also binds to these channels and, once labeled with [¹⁸F], can serve to detect areas of demyelination using positron emission tomography (PET). Given the potential of these molecules as therapeutic and imaging agents, we set out to investigate four new 4AP derivatives and their structure-activity relationships.

Prior work on the structure-activity relationship studies of 4AP derivatives has shown that small modifications on the 3 position are permitted and that the in vivo potency is highly correlated with the pKₐ. 4AP and derivatives are basic compounds that exist in the protonated or neutral form depending on the pH of the medium (Fig. 1). The protonated form mimics a large K⁺ ion and is required to block the channel, while the neutral form is required for the drug to get across the blood-brain barrier (BBB). In addition, the pharmacokinetic properties are largely dependent on the lipophilicity and pKₐ of these molecules. For example, 4AP has a pKₐ of 9.6, resulting in high potency but slow penetration into the CNS, which explains why a slow release formulation is required for therapy. 3F₄AP, on the other hand, has a pKₐ of 7.6 which results in a faster CNS penetration, which is favorable for PET imaging. Additional aminopyridine derivative examples include 3, 4-diaminopyridine (3, 4-DAP), a potent Kᵥ channel blocker with low BBB permeability, used clinically...
for Lambert-Eaton syndrome, a disorder of peripheral nervous system, and 4-aminopyridine-3-methanol (4AP3MeOH) which has been shown to enhance conduction in laboratory models of spinal cord injury and MS, but that has minimal permeability to the BBB and requires intrathecal administration.

Given the correlations between the pKₐ, lipophilicity and molecular size with in vivo activity, we hypothesized that the derivatives 3-methyl-4-aminopyridine (3Me4AP), 3-methoxy-4-aminopyridine (3MeO4AP), 3-trifluoromethyl-4-aminopyridine (3CF₃4AP) and 2-trifluoromethyl-4-aminopyridine (2CF₃4AP) would be permeable to the CNS and be suitable candidates for therapy and/or imaging (Fig. 2A). These molecules are particularly interesting as potential PET radioligands since they are amenable to labeling with ¹¹C, which provides
some advantages over $^{18}$F-labeled radioligands. While $^{11}$C has a significantly shorter half-life compared to $^{18}$F (20 vs. 110 min) limiting its use to sites with a cyclotron, $^{11}$C-labeled tracers tend to be easier to radiolabel and their short half-life allows for multiple scans on the same subject and day; an important advantage during tracer development and validation. In fact, methods to produce radiolabeled $[^{11}$C]$3$MeO4AP, $[^{11}$C]$2$CF3AP and $[^{11}$C]$3$CF34AP have recently been communicated but evidence that these compounds are able to bind to K$_v$ channels is lacking.

Results

Basicity and lipophilicity of 4AP analogs. The chemical structures and the abbreviations of the 4AP analogs studied are shown in Fig. 2A. Table 1 shows the p$_{K_a}$ values of these compounds in order of decreasing basicity. As shown on the table: 4AP, 3Me4AP, and 3MeO4AP are more basic with p$_{K_a}$ values higher than 9, while 3F4AP, 3CF4AP and 2CF34AP are less basic with p$_{K_a}$ values lower than 8. This indicates that the former are mostly protonated at physiological pH, while the latter exist both in the protonated and neutral forms at physiological pH.

In terms of lipophilicity 4AP, 3Me4AP, and 3MeO4AP were found to have octanol/water partition coefficient values at pH 7.4 of $-1.48, -1.23$ and $-0.76$ (Table 1). This indicates that these compounds preferably partition in the aqueous layer and may have lower penetration of the BBB by passive diffusion. In contrast, 3F4AP, 3CF4AP and 2CF34AP show partition coefficient values of 0.41, 1.48 and 0.91 (Table 1) indicating that these compounds preferably partition in the octanol layer and may have a faster permeation of the BBB. In fact, 4AP is known to have a slow penetration of the BBB while 3F4AP has a fast BBB penetration.

Both p$_{K_a}$ and logD trends can be rationalized by the electron-donating or electron-withdrawing nature of the substituent. As the electron-withdrawing strength of the group increases, the dipole of the pyridine nitrogen decreases resulting in a more acidic proton. When comparing the 2CF34AP with 3CF34AP, substitution in the 3 position of 4AP are permitted, whereas large modifications significantly diminish the potency of blockage.

Blocking capacity of 4AP analogs. K$^+$ currents were measured in Xenopus oocytes expressing the commonly studied voltage-gated K$^+$ channel Shaker from D. melanogaster. In order to determine the relative blocking capacity, each drug was applied to the same oocyte at increasing concentrations ranging from 1 to 10,000 μM. Then, the relative current was determined as the ratio between the maximal amplitude of the K$^+$ current in the absence and in the presence of each drug. Figure 2B shows five representative K$^+$ current traces from Shaker elicited at 40 mV, before and after addition of 1,000 μM of each drug. Figure 2C shows the relative current as a function of the concentration of each 4AP analog tested. The Hill equation (Eq. 1) was fitted to the dose-response curves and used to calculate the IC$_{50}$ for each drug at pH 7.4. Hill parameters are summarized in Table 1. Here, the limiting factor is the permeability of the drug through the oocyte membrane at low pH. In the case of the compounds with lower p$_{K_a}$, these drugs are able to permeate through the membrane even at low pH and the limiting factor is the fraction of protonated or active form of the drug.

Dependence of the blocking on pH. Since the canonical mechanism describes that only positively-charged molecules can block the channel and protonation of the drug is dependent on the pH, we studied the blocking of the channel (IC$_{50}$) at pH 6.8, 7.4 and 9.1. To avoid a gradient in pH, both internal and external solutions were replaced.

Panels A to E of Fig. 3 show the effects on the relative current as a function of concentration for each 4AP derivative at the different pH values. Interestingly, the analogs with high p$_{K_a}$ (4AP, 3Me4AP and 3MeO4AP) showed higher blocking ability (lower IC$_{50}$) at higher pH, whereas the analogs with lower p$_{K_a}$ showed higher blocking ability at lower pH. Since 4AP and derivatives bind from the intracellular side, we hypothesize that in the case of the compounds with high p$_{K_a}$, the limiting factor is the permeability of the drug through the oocyte membrane at low pH. In the case of the compounds with low p$_{K_a}$, these drugs are able to permeate through the membrane even at low pH and the limiting factor is the fraction of protonated or active form of the drug. Table 2 summarizes the IC$_{50}$ values of each analog at different pH values calculated by fitting the Hill equation to the data.

### Table 1. p$_{K_a}$ and logD (at pH = 7.4) values of the 4AP analogs.

| Drug     | p$_{K_a}$    | logD   |
|----------|--------------|--------|
| 3Me4AP   | 9.82 ± 0.06  | -1.232 ± 0.008 |
| 4AP      | 9.58 ± 0.07  | -1.478 ± 0.014 |
| 3MeO4AP  | 9.18 ± 0.02  | -0.76 ± 0.03  |
| 3F4AP    | 7.65 ± 0.15  | 0.414 ± 0.002 |
| 2CF34AP  | 7.0 ± 0.4    | 0.906 ± 0.006 |
| 3CF34AP  | 7.17 ± 0.04  | 1.484 ± 0.009 |

Dependence of the blocking on pH. Since the canonical mechanism describes that only positively-charged molecules can block the channel and protonation of the drug is dependent on the pH, we studied the blocking of the channel (IC$_{50}$) at pH 6.8, 7.4 and 9.1. To avoid a gradient in pH, both internal and external solutions were replaced.

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Dependence of the blocking on voltage. It is known that blocking of K<sup>+</sup> channels by 4AP involves sequential voltage-dependent rearrangements of the protein<sup>31</sup>. During this process, K<sub>V</sub> channels must transition to the open conformation before 4AP can bind to its site inside the channel pore. Therefore, it is expected that the blocking of the studied 4AP derivatives will be voltage-dependent as it has been shown for 4AP<sup>48</sup>. For this reason, we studied the IC<sub>50</sub> at several voltage values. We recorded K<sup>+</sup> currents from Shaker channel in the range of voltage from −100 to 50 mV (see Fig. S1) and calculated the IC<sub>50</sub> of each drug and voltage as described above. Panels A to E of Fig. 4 show the measured relative current as a function of the concentration of each 4AP analog at several voltage values. For clarity, only representative curves at +10, +30 and +50 mV are shown. From Fig. 4, it can be observed that the calculated IC<sub>50</sub> for each 4AP analog increased with voltage (in μM): from 200 to 350 for 4AP, from 160 to 304 for 3F<sub>4</sub>AP, from 37 to 50 for 3Me<sub>4</sub>AP, from 310 to 992 for 3MeO<sub>4</sub>AP, and from 690 to 1150 for 3CF<sub>3</sub>4AP. These results confirm that blocking of the channel is voltage-dependent and that it is more difficult to block the passage of K<sup>+</sup> ions at higher voltages than at lower voltages.

Furthermore, this experiment allowed us to calculate the fractional distance through the membrane electrical field that each molecule travels to bind to its site during the experiment<sup>49</sup>. The Woodhull equation (Eq. 2) describes that the relationship between IC<sub>50</sub> and voltage is dependent on δ. By fitting the IC<sub>50</sub> vs. V curves shown in Fig. 4D to the Woodhull equation, we were able to calculate δ for each 4AP analog. The fitted parameters from this analysis are shown on Table 3. Since δ values varied between 0.41 and 0.56, we conclude that these molecules have to travel approximately across one half of the membrane electric field. The similarity of the δ values between 4AP and the other derivatives suggests that these drugs share a common binding site.

### Table 2. IC<sub>50</sub> values of 4AP analogs at 0 mV: Hill parameters.

| Drug | pH 6.8 IC<sub>50</sub> (μM) | 95% C.I. (μM) | n | pH 7.4 IC<sub>50</sub> (μM) | 95% C.I. (μM) | n | pH 9.1 IC<sub>50</sub> (μM) | 95% C.I. (μM) | n |
|------|-----------------|-------------|---|-----------------|-------------|---|-----------------|-------------|---|
| 4AP  | 210             | 176–244     | 4 | 199             | 190–207     | 6 | 38              | 33–44       | 4 |
| 3F<sub>4</sub>AP | 82             | 76–88       | 5 | 160             | 140–179     | 5 | 185             | 144–226     | 4 |
| 3Me<sub>4</sub>AP | 66             | 56–76       | 6 | 39              | 36–42       | 4 | 15              | 13–17       | 5 |
| 3MeO<sub>4</sub>AP | 357            | 325–390     | 4 | 355             | 327–382     | 4 | 492             | 446–538     | 5 |
| 3CF<sub>3</sub>4AP | 249            | 150–348     | 5 | 690             | 633–747     | 6 | 2188            | 1317–3057   | 4 |
| 2CF<sub>3</sub>4AP | 10,448         | 7,909–12,985| 4 | 11,903          | 5,111–18,695| 4 | 18,215          | 2776–33,653 | 2 |

### Figure 3. IC<sub>50</sub> at 0 mV and pH dependence. Relative current vs. concentration at 0 mV at pH of 6.8 (red), 7.4 (gray) and 9.1 (blue) of: (A), 4AP, (B), 3F<sub>4</sub>AP, (C), 3Me<sub>4</sub>AP, (D), 3MeO<sub>4</sub>AP and, (E), 3CF<sub>3</sub>4AP, (F), 2CF<sub>3</sub>4AP. Continuous lines in panels A to E represent the fits with the Hill equation (Eq. 1). Hill parameters are summarized on Table 2.
Discussion

Screening the ability of fluorinated analogs of 4AP to block K⁺ currents in *Xenopus* oocytes expressing Shaker K⁺ channel facilitated the identification of 3F4AP as a potential PET radioligand for demyelination. Subsequent labeling of 3F4AP with ¹⁸F and PET imaging studies in rodent models of MS confirmed the capacity of this compound to detect demyelinated lesions using PET. This compound is in progress to clinical studies and has the potential to advance the imaging of demyelinating diseases.

In PET tracer development, similar to drug development, it is useful to explore multiple derivatives and compare their properties. This process provides confirmation of the target and may result in radioligands with enhanced characteristics. In addition, it is convenient to have ¹⁸F and ¹¹C versions of the same tracer, as each radiolabeling method provides certain advantages that the other lacks. For example, while ¹⁸F-labeled tracers can be used in sites remote from a cyclotron because of its longer half-life, ¹¹C-labeled tracers enable multiple scans (be it more than one ¹¹C-scan or a combination of ¹¹C/¹⁸F-scans) on the same subject and the same day.

Here, we studied the efficiency of blocking K⁺ currents of four novel 4AP analogs under steady-state, namely: 3-methyl-4-aminopyridine (3Me4AP), 3-methoxy-4-aminopyridine (3MeO4AP), 3-(trifluoromethyl)-4-aminopyridine (3CF₃4AP), 2-(trifluoromethyl)-4-aminopyridine (2CF₃4AP) (see chemical structures in Fig. 1A). We selected these compounds as they are predicted to be permeable to the BBB and are amenable to labeling with ¹¹C. During our studies, we found that all of these compounds are able to block Shaker (homolog of mammalian Kv1.2) channel albeit with different potencies (IC₅₀ ranging from 160 to 12,000 μM). Specifically, 3Me4AP was found to be ~7 times more potent than 4AP and 3F4AP, 3MeO4AP and 3CF₃4AP were about 3–4 times less potent and 2CF₃4AP was around 60 times less potent. This study is timely given that methods to label 3MeO4AP and 2- and 3-CF₃4AP have recently been reported but the capacity of these compounds to bind to the K⁺ channels is unknown. Thus, this study may help decide which compounds to take for further imaging development.

Table 3. IC₅₀ and δ values of 4AP analogs at 0 mV: Woodhull parameters. *Mean values are given ± SD.

| Drug       | IC₅₀ (μM) | δ       | n |
|------------|----------|---------|---|
| 4AP        | 155 ± 12 | 0.41 ± 0.08 | 6 |
| 3F4AP      | 122 ± 4  | 0.46 ± 0.10 | 5 |
| 3Me4AP     | 21 ± 1   | 0.43 ± 0.10 | 4 |
| 3MeO4AP    | 329 ± 17 | 0.56 ± 0.02 | 4 |
| 3CF₃4AP    | 516 ± 51 | 0.46 ± 0.10 | 6 |

Figure 4. Voltage-dependence of the IC₅₀ for each 4AP analog. Relative current as a function of concentration of (A) 4AP, (B) 3F4AP, (C) 3Me4AP, (D) 3MeO4AP, and (E) 3CF₃4AP, obtained at different values of voltage. For clarity only the curves obtained at 10, 30 and 50 mV are shown. Solid lines represent the fits of the data with the Hill equation (Eq. 1). (F) IC₅₀ vs. voltage curves of 4AP analogs determined in the range of voltage from 10 to 50 mV. IC₅₀ values were obtained from the analysis of the data of the panels A to E. Dashed lines represent the fits with the Woodhull model (Eq. 2). Woodhull parameters δ and IC₅₀ at V = 0 mV are shown on Table 3.
The values obtained in this study represent the potency of the tested drugs towards Shaker channel expressed in Xenopus oocytes, which as discussed by A. L. Goldin is typically lower than the potency measured in mammalian cells or even native tissues. These differences in potency typically arise from the large number of interspecies around the oocyte, the presence of the vitelline membrane, the follicles surrounding the membrane, and the intracellular yolk structures, which may act as a sink for the drug. Nevertheless, Goldin also states that the relative efficacies of drugs against channels expressed in Xenopus oocytes are generally representative of those in native tissues. Thus, this study provides strong evidence that the in vivo affinity of the inhibitors will be as follows: 3MeO4AP > 3F4AP > 4AP > 3Me4AP > 3CF44AP > 2CF44AP.

Furthermore, the physicochemical characterization of these compounds in terms of basicity and lipophilicity provided here are also informative for future prioritization in drug or tracer development as many critical properties for successful pharmaceuticals and radiopharmaceuticals are related to these properties including brain penetration, clearance rate and metabolic stability.

Finally, we studied the voltage- and pH-dependence of the compounds blocking ability, as these results can provide valuable mechanistic information. From the pH-dependence, we observed that the compounds with lower \( pK_a \) (i.e., 3F4AP, 3CF44AP and 2CF44AP) are less active at basic pH, presumably because not enough of the protonated/active form is present. At the same time, the compounds with higher \( pK_a \) (i.e., 4AP, 3Me4AP and 3MeO4AP) are more active at basic pH as it facilitates membrane permeability. Regarding the voltage-dependence, we found that blocking is more effective at lower voltages than at higher voltages, as the voltage provides a greater driving force to the \( K^+ \) ions than to the drugs. From the voltage-dependence analyses, we were able to estimate using the Woodhull equation that these drugs travel about 50% of the membrane electrical field in order to bind. This is consistent with prior studies about AP binding site and strongly suggests that these drugs share a common binding site.

In summary, we have characterized four novel derivatives of 4AP as potential candidates for therapy and imaging. The physicochemical and pharmacological properties described here will be useful for selecting the compounds with most potential and to explain the differences in terms of drug efficacy and tracer sensitivity.

**Methods**

**\( pK_a \) determination.** The \( pK_a \) of each compound was measured by acid titration. Briefly, 5 mg of each compound were dissolved in 5 mL of water and titrated with 0.01 M HCl solution (0.01 M NaOH solution for age provides a greater driving force to the \( K^+ \) voltage-dependence, we found that blocking is more effective at lower voltages than at higher voltages, as the volt-

**Partition coefficient determination.** The octanol-water partition coefficient (logD) at pH 7.4 was determined using a modified version of the shake flask method. Briefly, PBS (900 µL), 1-octanol (900 µL) and a 10 mg/mL aqueous solution of each compound (2 µL) were added to a 2 mL HPLC vial. The compounds were partitioned between the layers via vortexing and centrifuged at 1,000 g for 1 min to allow for phase separation. A portion (10 µL) was taken from each layer (autoinjector was set up to draw volume at two different heights) and analyzed by HPLC. The relative concentration in each phase was determined by integrating the area under each peak and comparing the ratio of the areas from the octanol and aqueous layers. A calibration curve was performed to ensure that the concentrations detected were within the linear range of the detector. This procedure was repeated 4 times for each compound.

**Synthesis of Shaker \( K^+ \) channel RNA.** A sample of cDNA clone encoding for Shaker voltage-gated \( K^+ \) channel from *D. melanogaster* with inactivation removed was generously provided by the laboratory of Prof. Francisco Bezanilla at The University of Chicago. The DNA was amplified, linearized with Not I enzyme (New England Biolabs, Inc., Ipswich, MA, USA) and transcribed *in vitro* using the T7 promoter mMESSAGE cRNA kit (Ambion, Austin, Tex., USA).

**Expression of Shaker \( K^+ \) channels in Xenopus oocytes.** Shaker channel was heterologously expressed in *Xenopus laevis* oocytes. Only mature *Xenopus laevis* frogs (Aquanimals SA de CV, Queretaro, Mexico) were used as oocytes suppliers. A volume of 1 – 3 mL from the ovary lobes was extracted via survival surgery under anesthesia. All methods involving live animals were performed in accordance with relevant guidelines and regulations and with the approval of the Comité Institucional del Cuidado y Uso de Animales en el Laboratorio (CICUAL-CUCEI-UDG). Subsequently, oocytes were isolated with the treatment of collagenase type II and the intracellular yolk structures, which may act as a sink for the drug. Nevertheless, Goldin also states that the relative efficacies of drugs against channels expressed in Xenopus oocytes are generally representative of those in native tissues. Thus, this study provides strong evidence that the in vivo affinity of the inhibitors will be as follows: 3MeO4AP > 3F4AP > 4AP > 3Me4AP > 3CF44AP > 2CF44AP.

**Recording of \( K^+ \) currents using cut-open voltage clamp.** All chemical compounds for this study were acquired from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA) and Chem-Impex International (Chem-Impex International, Inc. Wood Dale, IL, USA) unless otherwise indicated. Electrophysiology measurements were conducted using the methodology of cut-open oocyte voltage clamp (COVC) for COVC procedures, the internal recording solution contained (in mM): 120 KOH, 2 EGTA, and 20 HEPES. The external recording solution was composed (in mM) by: 12 KOH, 2 Ca(OH)2, 105 NMDG (N-methyl-D-glucamine)-methylsulfonate (MES), and 20 mM HEPES. For measurements carried out at pH 6.8 and 7.4, the pH of both solutions was adjusted with MES. For measurements carried out at pH = 9.1, HEPES was replaced by 2-(cyclohexylamino)ethanesulfonic acid (CHES).
To quantify the effects upon K+ currents of 4AP and 4AP analogs, oocytes that successfully expressed the Shaker channel were voltage-clamped in a COVC station. K+ currents were recorded in the same oocyte, first in absence (\(I_R\)) and then in presence of each drug (\(I_I\)). \(I_R\) was elicited by depolarizing the oocyte membrane with a voltage protocol that consisted in steps of 50 ms from −100 to 50 mV in increments of 10 mV. Then, \(I_I\) was achieved by replacing the external solution (top and guard chambers) with a solution containing increasing concentrations of each drug (4AP), 3-fluoro-4-aminopyridine (3F4AP), 3-methyl-4-aminopyridine (3Me4AP), 3-methoxy-4-aminopyridine (3MeO4AP), 2-trifluoromethyl-4-aminopyridine (2CF34AP) and 3-trifluoromethyl-4-aminopyridine (3CF34AP). Because 4AP and 4AP analogs block the K+ channels in its open conformation, the oocytes were pulsed 5 to 10 times at 10 mV (1 min pulse) until a stable \(I_I\) was achieved. The integrity and stability of each oocyte were continuously monitored throughout the experiment.

Ionic currents were amplified and digitized with the Oocyte Clamp Amplifier CA-1A (Dagan Corporation, Minneapolis, MN, USA) and the USB-1604-HS-2AO Multifunction Card (Measurement Computing, Norton, MA, USA), respectively, and controlled with the GpPatchMC64 program (Department of Anesthesiology, UCLA, Los Angeles, CA, USA) via a PC. Data were sampled at 100 kHz and filtered at 10 kHz. All the experiments were performed at room temperature (21–23°C).

**Electrophysiology data analysis.** Ion currents recordings were analyzed with Analysis (Department of Anesthesiology, UCLA, Los Angeles, CA, USA) and OriginPro 8 (OriginLab Corporation, Northampton, MA, USA.) programs. The half-maximal inhibitory concentration of 4AP and 4AP analogs (\(IC_{50}\)) was determined by fitting the relative current (\(I_{rel} = I_I/I_R\)) as a function of the cumulative concentration of each drug ([X]) with the Hill equation:

\[
I_{rel} = \frac{I_{max} - I_{min}}{1 + 10^{\delta \log I_{50} - \log(X/e)}}
\]

where \(I_{max}\) and \(I_{min}\) are the maximal and minimal value of \(I_{rel}\) respectively, and \(h\) is the Hill coefficient, which was typically 1 ± 0.1 under our experimental conditions.

The voltage-dependence of blocking by 4AP and 4AP analogs was analyzed in terms of the \(IC_{50}\) as a function of \(V (IC_{50}(V))\). \(IC_{50}(V)\) curves were fitted with a one-step model of inhibition, which allowed to determine the fractional distance through the membrane electrical field (\(\delta\)) that each 4AP analog has to cross to reach its binding site:

\[
\log IC_{50} = \log IC_{50}(V=0) + \frac{zFV}{2.303RT}\]

where \(IC_{50}(V=0)\) is the value of \(IC_{50}\) at \(V=0\) mV, \(F\) is the Faraday constant, \(R\) is the gas constant, \(T\) is the ambient temperature, and \(z\) is the apparent charge. Mean values of data ± standard deviation (s.d.) are given or plotted and the number of experiments is denoted by \(n\). The 95% of confidence interval (\(IC_{50}\)) is denoted as [Upper limit-Lower limit]; where Upper limit = \(10^{\log IC_{50} + 2.576\delta}\) and Lower limit = \(10^{\log IC_{50} - 2.576\delta}\).

**Data availability**

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions
S.R.R. performed the COVC experiments and analyzed the data. A.D.B. and K.M.R.T. performed the pK_a and logD experiments and analyzed the data. P.B. and J.E.S.R. conceived and supervised the project and wrote the manuscript. All authors revised the manuscript.

Competing interests
The University of Chicago has obtained patents related to the compounds described here where P.B. is listed as inventor (U.S. Patent: US10160695B2 and US9617215B2). Other authors declare no competing interests.

Additional information
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