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KCNQ5 activation is a unifying molecular mechanism shared by genetically and culturally diverse botanical hypotensive folk medicines

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Botanical folk medicines have been used throughout human history to treat common disorders such as hypertension, often with unknown underlying mechanisms. Here, we discovered that hypotensive folk medicines from a genetically diverse range of plant species each selectively activated the vascular-expressed KCNQ5 potassium channel, a feature lacking in the modern synthetic pharmacopeia, whereas nonhypotensive plant extracts did not. Analyzing constituents of the hypotensive *Sophora flavescens* root, we found that the quinolizidine alkaloid aloperine is a KCNQ-dependent vasorelaxant that potently and isoform-selectively activates KCNQ5 by binding near the foot of the channel voltage sensor. Our findings reveal that KCNQ5-selective activation is a defining molecular mechanistic signature of genetically diverse traditional botanical hypotensives, transcending plant genus and human cultural boundaries. Discovery of botanical KCNQ5-selective potassium channel openers may enable future targeted therapies for diseases including hypertension and KCNQ5 loss-of-function encephalopathy.

potassium channel | hypertension | herbal medicine

Plants are a rich source of bioactive compounds and have been utilized medicinally for millennia, as recorded in clay tablets from ancient Mesopotamia (1) and texts from ancient Egypt and China (2, 3). Archaeological evidence even suggests that Paleolithic hominins, in what is now Israel, utilized a variety of plants considered to be medicinal rather than food, including fennel seed, chamomile, and willow, as long as 800,000 y ago (4). Recently, traces of chamomile and willow were also discovered in the dental calculus of *Homo neanderthalensis*, suggesting herbal self-medication in this species 48,000 y ago (5, 6) (Fig. 1*A*).

In many instances, the molecular basis for therapeutic activity of botanical folk medicines remains unknown, despite being used for millennia and efficacy having been established in some cases by animal studies or even clinical trials. The modern approach to drug discovery often involves screening hundreds of thousands of compounds for efficacy on a target protein or cellular activity of interest. Lead compounds identified in this manner are then optimized by further chemical synthesis. Despite the availability of colossal libraries of compounds, some targets have remained resistant to discovery of small-molecule modulators.

One relatively intractable area has been the development of ion channel openers. Many ion channel antagonists are known, but there are much known fewer agonists, reflecting the fact that it is less challenging to block a channel pore than it is to positively modulate the channel activation machinery to facilitate opening. Chemical activation of the KCNQ (Kv7) subfamily of voltage-gated potassium (Kv) channels has been one particular focus, resulting in some success. The KCNQ subfamily comprises 5 genes, each encoding 6 transmembrane segment (S) pore-forming (α) subunits with a voltage sensing domain and a pore module (Fig. 1*B*). Four such α subunits tetramerize to form a functional potassium channel. KCNQ1 is a ubiquitous and

functionally diverse isoform expressed in cardiac myocytes, vascular smooth muscle, and many different epithelia throughout the body. Its functional diversity arises from coassembly with transmembrane β subunits from the KCNE subfamily; it is considered unlikely that KCNQ1 forms homomeric (KCNE-less) channels in vivo, as currents with the properties of homomeric KCNQ1 (known from heterologous expression studies) have not been detected in native cells (7). KCNQs 2–5 are expressed in neurons; channels formed by KCNQ2/3 heteromers, KCNQ3/5 heteromers, and possibly KCNQ2, KCNQ3, and/or KCNQ5 homomers generate the M-current, a muscarinic-receptor–inhibited subthreshold Kv current vital to regulating neuronal firing (8–12). KCNQ4 is essential for hearing and is expressed in auditory neurons and hair cells (13). KCNQ1, KCNQ4, and KCNQ5 are also expressed in vascular smooth muscle, where their activity regulates vascular tone (14).

Retigabine (ezogabine) is a first-in-class anticonvulsant that activates KCNQ2–5 isoforms, potentiating the M-current to dampen aberrant neuronal excitability (15). Retigabine binds to a channel pocket in which a specific S5 tryptophan residue (W265 in KCNQ3) essential for binding and/or channel activation is thought to be exposed (Fig. 1*C*). Retigabine does not activate KCNQ1, which lacks the equivalent S5 tryptophan (16). Other than this, retigabine shows limited isoform selectivity, displaying a 2- to 5-fold

Significance

Botanical folk medicines have been used by diverse human populations and cultures for several millennia. Many are still in use today, but the underlying molecular mechanisms often remain elusive. Here we report the discovery of a molecular mechanism linking diverse plant extracts used traditionally to lower blood pressure (hypotensives). All of the hypotensive plants tested activated the KCNQ5 vascular-expressed potassium channel, whereas nonhypotensives did not. For one hypotensive plant, we describe discovery of the active small molecule (aloperine) and demonstrate that it KCNQ-dependently relaxes blood vessels. The discovery opens up a new source of potential therapeutic drugs and explains the mechanism behind folk hypotensive medicines used by diverse populations for thousands of years.
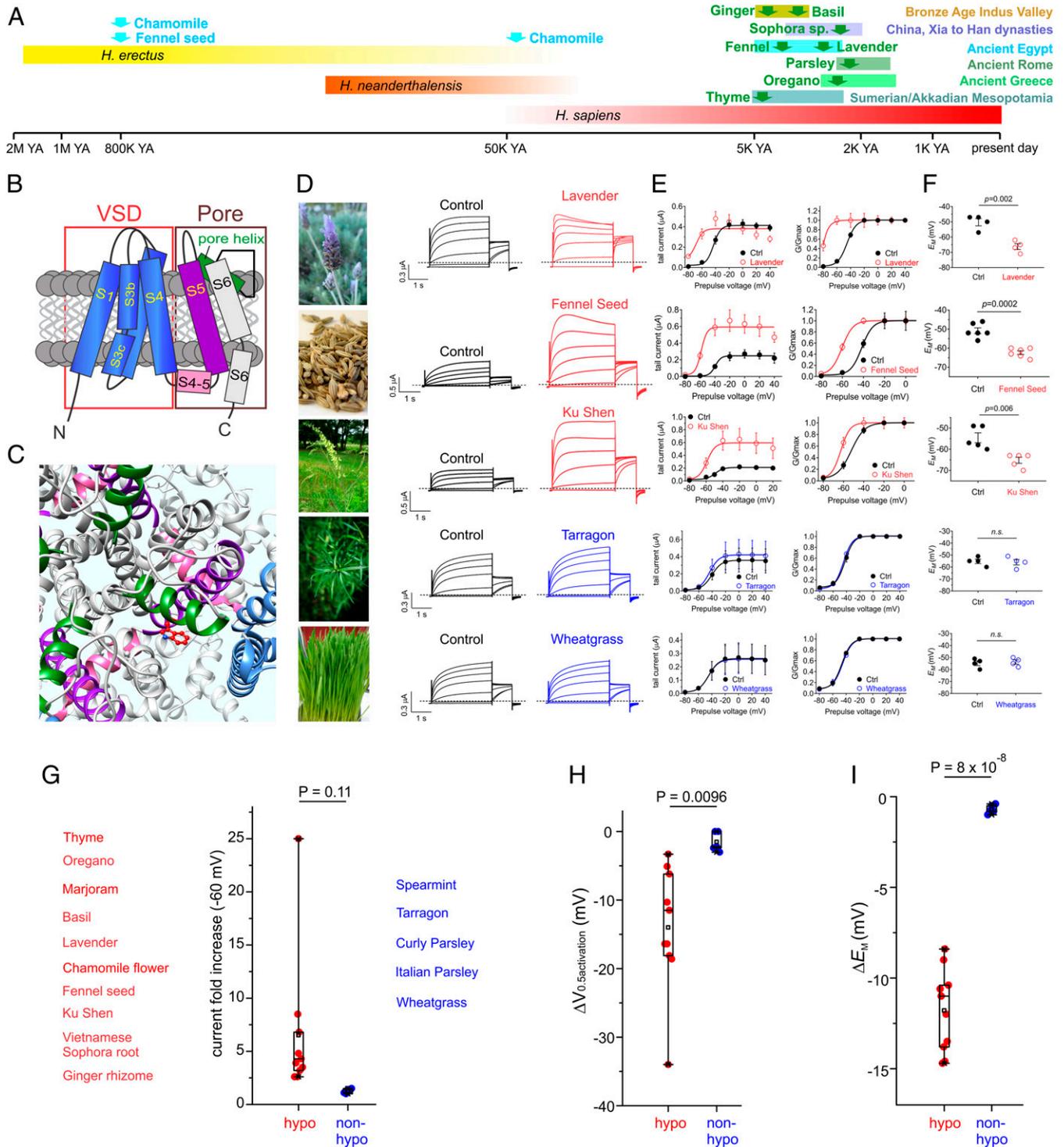
Fig. 1. KCNQ5 activation is a specific, shared feature of botanical hypotensive folk medicines. All error bars indicate SEM. (*A*) Approximate timeline of human use of the hypotensive plants examined in this study. YA, years ago. (*B*) Topological representation of KCNQ5 showing 1 of the 4 subunits that comprise a channel. VSD, voltage sensing domain. (*C*) Extracellular view of the chimeric KCNQ1/KCNQ5 structural model to highlight the anticonvulsant binding pocket (red; KCNQ5-W235) using the color coding as in *B*. (*D*) A subset of the plants screened in this study (*SI Appendix*, Fig. S2 shows full screening data). (*Left*) Images of the plants used (attributions provided in *SI Appendix*). (*Right*) Mean TEVC current traces for KCNQ5 expressed in *Xenopus* oocytes in the absence (control) or presence of 1% extract from the medicinal plants as indicated (*n* = 4 to 5). Dashed line here and throughout indicates zero current level. Red indicates plants previously reported to show hypotensive activity or used traditionally as hypotensives; blue indicates nonhypotensives or diuretic hypotensives (here and *E–I*). (*E*) Mean tail current (*Left*) and normalized tail currents (G/Gmax; *Right*) versus prepulse voltage relationships for the traces as in *D* (*n* = 4 to 5). (*F*) Effects of the hypotensive plant extracts shown in *D* on resting membrane potential (E_M) of unclamped oocytes expressing KCNQ5 (*n* = 4 to 5). (*G–I*) Scatter plots showing mean effects for the full screen (*SI Appendix*, Fig. S2) of hypotensive (red) versus nonhypotensive (blue) 1% plant extracts on (*G*) current at −60 mV, (*H*) V$_{0.5activation}$, and (*I*) E_M in oocytes expressing KCNQ5 (*n* = 4 to 8). Each point represents the mean data from 1 plant species.

higher potency for KCNQ3 versus KCNQ2, 4, and 5; the related ML213 behaves similarly. ICA-069673 is 20-fold more potent with respect to KCNQ2/3 versus KCNQ3/5 activation (17), but robustly activates KCNQ4 (18). ICA-27243 is >20-fold more potent at activating KCNQ2/3 compared to KCNQ4 (19). All 4 of the aforementioned compounds contain a carbonyl group considered important for interaction with KCNQ3-W265 and its equivalents in other KCNQs (20), while all but ML213 also bear fluorophenyl rings (*SI Appendix*, Fig. S1).

There are currently no reported KCNQ5-selective openers. KCNQ5 is widely expressed in the brain (21), airway epithelium (22, 23), retina (24), and auditory brainstem nuclei (25). In addition, KCNQ5 is required for normal regulation of vascular (26, 27), uterine (28), penile (29), and gastrointestinal (30) smooth muscle. In vascular smooth muscle cells, KCNQ5 forms heteromultimeric channels with KCNQ4 to control arterial tone at rest, with pharmacological blockade of these channels leading to vasoconstriction (31). Rare gain- or loss-of-function KCNQ5 gene variants (32) and KCNQ5 haploinsufficiency (33) cause severe intellectual disability and epileptic encephalopathy. KCNQ5 polymorphisms have also been associated with myopia (34), while KCNQ5 down-regulation is found in presbycusis (age-related hearing loss) (35). For most or all of these conditions, a KCNQ5-selective opener would be anticipated to have potential therapeutic activity.

Hypertension (high blood pressure) is currently highly prevalent and associated with high rates of cardiovascular disease and mortality in modern societies. Hypertension is typically diagnosed by directly measuring blood pressure. Historically, however, records dating back to 2600 BC indicate that "hard pulse disease"—considered to be equivalent to what is now termed hypertension—was diagnosed by assessing the pulse using gentle palpation. Hard pulse disease was historically treated in ancient Chinese, Ayurvedic, Greek, and Roman cultures variously by using venesection, acupuncture, leeches, and herbal medicines. More recently, the efficacy of many traditional herbal hypotensives has been borne out by animal studies and/or clinical trials (36–39).

Here, given the importance of KCNQ5 in the vasculature and the long history of medicinal plant use in treating hypertension, we adopted an alternative approach to identifying a KCNQ5-selective opener. We screened the extracts of several plants used traditionally to lower blood pressure (hypotensives) for KCNQ5 channel opening activity, hypothesizing that the vasodilatory action of hypotensives might arise from KCNQ5 activation. We discovered that, remarkably, KCNQ5-selective activation is a shared feature of a diverse range of botanical hypotensives, spanning human history and cultures and regardless of plant genus. Further investigation revealed that aloperine, a small molecule from the hypotensive root extract of the Chinese medicinal plant *Sophora flavescens*, exhibits KCNQ5-selective opening activity with submicromolar affinity and KCNQ-dependently relaxes mesenteric resistant arteries.

Results

Hypotensive Botanical Extracts Activate KCNQ5. We performed methanolic extractions (80% methanol/20% water) of 15 traditional medicinal plants spanning several clades and orders (Fig. 1*D* and *SI Appendix*, Fig. S2*A*). Of the 15 plants, 10 were chosen for their purported hypotensive properties: the leaves and flowers of fresh lavender (*Lavandula angustifolia*) and German chamomile (*Matricaria chamomilla*); leaves of fresh basil (*Ocimum basilicum*), oregano (*Origanum vulgaris*), marjoram (*Origanum majorana*), and thyme (*Thymus vulgaris*); whole fennel seed (*Foeniculum vulgare*); dried rhizome of ginger (*Zingiber officinale*); dried roots of shrubby Sophora (*Sophora flavescens*; termed Ku Shen in Chinese traditional medicine); and dried Vietnamese Sophora (*Sophora tonkinensis*) root (VSR) and rhizome. We classified the plants as hypotensive based on historic and/or current usage to purportedly lower blood pressure,

coupled with published clinical, preclinical, and/or ex vivo tissue studies demonstrating the capacity of the extracts to lower blood pressure and/or vasorelax (40–52).

As controls, we tested spearmint (*Mentha spicata*), tarragon (*Artemisia dracunculus*), wheatgrass (*Triticum aestivum*), and Italian parsley (*Petroselinum crispum neapolitanum*), which are not traditionally used or generally considered to act as hypotensives (53, 54). We also tested curly parsley (*Petroselinum crispum*), which may have hypotensive properties arising from diuretic effects, but which would not be expected to act via vascular KCNQ5 channels and is thought to instead involve inhibition of renal $Na^+/K^+ATPases$ (55, 56). After removing the methanol from each sample to leave aqueous solutions, we screened for KCNQ5 activation by a 1/100 dilution of each of the extracts using 2-electrode voltage clamp electrophysiology of KCNQ5 expressed in *Xenopus laevis* oocytes.

Remarkably, 1% extracts of all of the hypotensive plants, except for the diuretic curly parsley, exhibited KCNQ5-opening activity, while the nonhypotensives and curly parsley did not (Fig. 1 *D* and *E* and *SI Appendix*, Fig. S2 *B–D* and Table S1). This opening effect was manifested as a negative shift in the voltage dependence of KCNQ5 activation ($V_{0.5activation}$) and, for hypotensive plants thyme, oregano, basil, fennel seed, and *S. flavescens*, increased KCNQ5 peak current magnitude (Fig. 1 *D* and *E* and *SI Appendix*, Fig. S2 *B* and *C*). Consistent with the negative shift in voltage dependence, only the hypotensive plant extracts hyperpolarized the resting membrane potential (E_M) of oocytes expressing KCNQ5 (Fig. 1*F* and *SI Appendix*, Fig. S2*D*). Thus, KCNQ5-opening activity was strongly enriched in hypotensive versus nonhypotensive plant extracts, as quantified by fold increase in current at −60 mV, $\Delta V_{0.5activation}$, and ΔE_M (Fig. 1 *G–I*).

Hypotensive Botanical Extracts Do Not Activate KCNQ2/3. Heteromeric KCNQ2/3 complexes are the predominant KCNQ channel type in mammalian brain. KCNQ2–5 openers reported to date open KCNQ2/3 preferentially versus KCNQ5 channels (17–19). We therefore next tested the botanical extracts for KCNQ2/3 activity. Strikingly, none of the hypotensive plant extracts exhibited significant KCNQ2/3 opening activity, aside from thyme, which induced a small negative $\Delta V_{0.5activation}$ (*SI Appendix*, Fig. S3 *A* and *B*), suggesting thyme extract may contain a less isoform-specific KCNQ opener than the other hypotensive herbs. Consistent with the lack of activity, neither did any of the extracts (including thyme) hyperpolarize the membrane potential of oocytes expressing KCNQ2/3 (*SI Appendix*, Fig. S3*C*). Similarly, the nonhypotensives we tested also did not open KCNQ2/3 channels (*SI Appendix*, Fig. S3 *D* and *E*) or KCNQ2/3-dependently hyperpolarize E_M (*SI Appendix*, Fig. S3*F*). Therefore, in contrast to previously reported KCNQ-opening small molecules, the hypotensive botanical extracts exhibited unique KCNQ5 selectivity among neuronal KCNQ isoforms with respect to activation, when quantified as fold increase in current at −60 mV (*SI Appendix*, Fig. S3*G*), $\Delta V_{0.5activation}$ (*SI Appendix*, Fig. S3*H* and Table S2), or ΔE_M (*SI Appendix*, Fig. S3*I*). The data also demonstrate the specificity of the effects of the hypotensive plant extracts: if effects were arising from endogenous oocyte factors, they would occur regardless of the expressed channel isoform.

Identification of a KCNQ5-Activating Small Molecule from *S. flavescens*. To identify possible active components within the hypotensive botanical extracts, we focused here on *S. flavescens* root extract (Fig. 2*A*), which is used in traditional Chinese medicine and it is documented to exhibit hypotensive and vasorelaxant properties (57, 58). Here, *S. flavescens* root extract exhibited both voltage-dependent and voltage-independent KCNQ5 opening activity yet had no effect on KCNQ2/3 (Fig. 1 and *SI Appendix*, Fig. S3). We tested
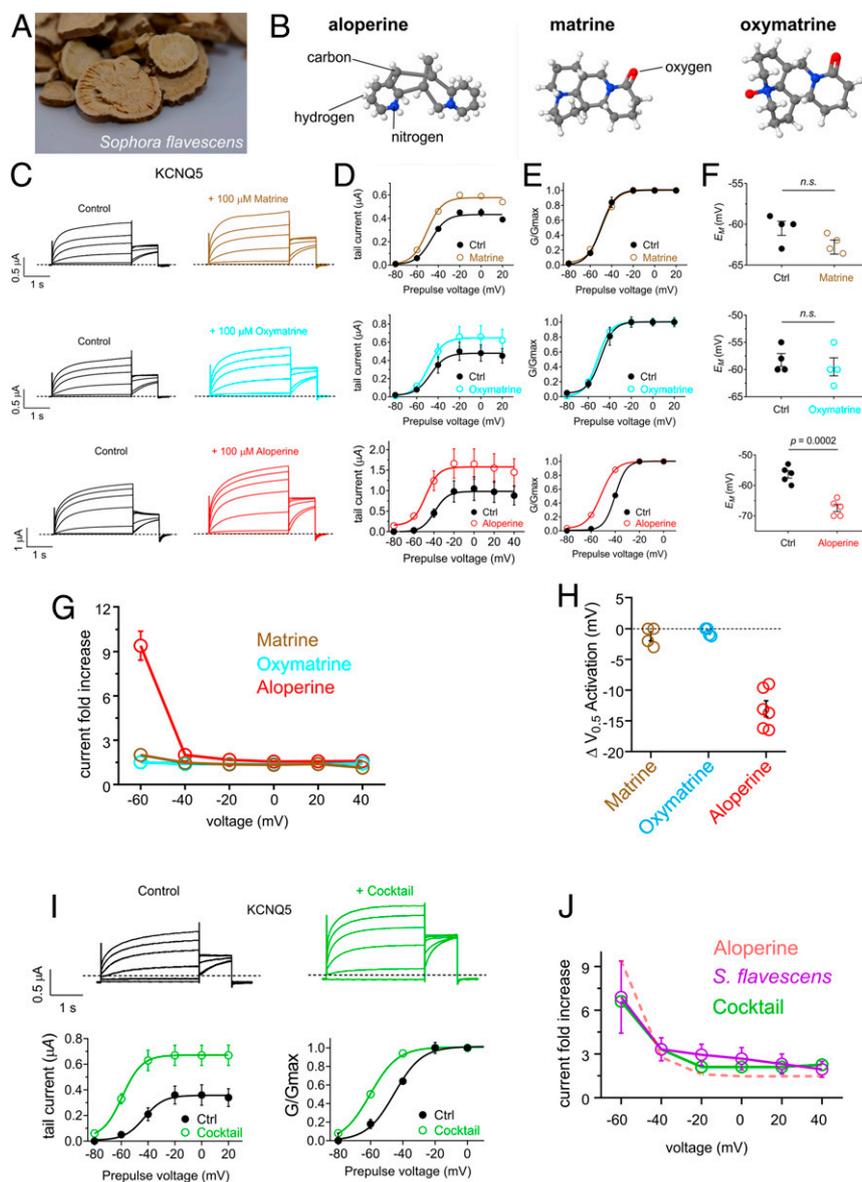
Fig. 2. The *S. flavescens* alkaloid aloperine activates KCNQ5. All error bars indicate SEM. (*A*) Image of dried *S. flavescens* root slices used in this study. (*B*) Chemical structures of aloperine, matrine, and oxymatrine plotted using Jmol. (*C*) Mean TEVC current traces showing effects of aloperine, matrine, and oxymatrine (all 100 μM) individually on KCNQ5 expressed in *Xenopus* oocytes (*n* = 4 to 6). (*D*) Mean tail current versus prepulse voltage relationships for the traces as in *C* (*n* = 4 to 6). (*E*) Mean normalized tail currents (G/Gmax) versus prepulse voltage relationships for the traces as in *C* (*n* = 4 to 6). (*F*) Effects of aloperine, matrine, and oxymatrine (100 μM) individually on resting membrane potential (E_M) of unclamped oocytes expressing KCNQ5 (*n* = 4 to 6). n.s., not statistically significant (*P* > 0.05). (*G*) Voltage dependence of effects of aloperine, matrine, and oxymatrine (100 μM) individually on KCNQ5 (from traces as in *C*; *n* = 4 to 6). (*H*) Mean effects of aloperine, matrine, and oxymatrine (100 μM) individually on KCNQ5 $V_{0.5activation}$ (from traces as in *C*; *n* = 4 to 6). (*I*) Mean effects of aloperine, matrine, and oxymatrine (each 1 μM) combined ("Cocktail") on KCNQ5 expressed in *Xenopus* oocytes (*n* = 5). (*Upper*) Mean TEVC traces; (*Lower Left*) mean tail current versus prepulse voltage relationships; (*Lower Right*) mean normalized tail currents (G/Gmax) versus prepulse voltage relationships. (*J*) Comparison of effects of aloperine (100 μM), cocktail, and *S. flavescens* extract (1%) on KCNQ5 activity, quantified as current fold change versus voltage (*n* = 5 to 6).

3 alkaloids previously identified in *S. flavescens* root extract: aloperine, matrine, and oxymatrine (Fig. 2*B*). Matrine and oxymatrine had relatively small effects on KCNQ5 activity (Fig. 2 *C* and *D*) and no effect on KCNQ5 $V_{0.5activation}$ (Fig. 2*E*). In contrast, the quinolizidine alkaloid aloperine (100 μM) robustly activated KCNQ5 (Fig. 2 *C* and *D*) and shifted KCNQ5 $V_{0.5activation}$ by −13.5 mV (Fig. 2 *C–E* and *SI Appendix*, Table S3). Consistent with the observed effects on KCNQ5 gating, aloperine, but not matrine or oxymatrine, hyperpolarized the E_M of oocytes expressing KCNQ5 (Fig. 2*F*). Aloperine induced the greatest increase in KCNQ5 current at −60 mV (Fig. 2*G*), explaining its hyperpolarizing effect on $\Delta V_{0.5activation}$ (Fig. 2*H*) and E_M (Fig. 2*F*).

We confirmed the presence of aloperine in the *S. flavescens* extract that we used for functional screening by using GC/MS (*SI Appendix*, Fig. S4). Next, using LC/MS, we found that the undiluted *S. flavescens* extract contains at minimum 4.71 ± 0.21 μM aloperine (*n* = 4). This value is a conservative, calculated minimum content for aloperine in the original extract. It is likely an underestimate because aloperine breaks down in water, and because a second peak in the extract may arise from aloperine, but we could not definitively make this assignment (*SI Appendix, Materials and Methods* and Fig. S5).

Aloperine effects were largely reminiscent of the effects of *S. flavescens* root extract on KCNQ5, but exhibited greater voltage

dependence than the extract (Figs. 1 *D–I* and 2*G*). Given the relatively voltage-independent and subtle effects of matrine and oxymatrine individually on KCNQ5 activity, we next tested the combination of aloperine, matrine, and oxymatrine (each at 1 μM). The combination ("cocktail") better recapitulated the effects of the *S. flavescens* root extract on KCNQ5 activity than did aloperine alone, increasing current potentiation at the less negative voltages (Fig. 2 *I* and *J*). We conclude that aloperine is the predominant KCNQ5-activating component in *S. flavescens* root extract, while matrine and oxymatrine (and possibly other unknown components) also have minor activating effects that contribute to recapitulating the effects of the whole extract.

KCNQ5 is thought to form heteromeric complexes with KCNQ3 in the brain (and was previously demonstrated to when the 2 were coexpressed in *Xenopus* oocytes [21]) and KCNQ4 in the vasculature, although the proportion and relative physiological significance of homomeric KCNQ5 versus heteromeric complexes containing KCNQ5 in either tissue is not known. Here, matrine and oxymatrine individually had no effect on KCNQ3/5, while the effects of aloperine were 2-fold less (quantified as current potentiation at −60 mV) than for homomeric KCNQ5. The mixture of all 3 compounds had a slightly lesser effect on KCNQ3/5 versus homomeric KCNQ5 (*SI Appendix*, Fig. S6 *A–F* and Tables S5 and S6). Both aloperine and the mixture were able to KCNQ3/5-dependently shift the oocyte resting membrane potential (*SI Appendix*, Fig. S6*G*), as we also observed for KCNQ5.

To examine KCNQ4/5 heteromers, we first ascertained whether KCNQ5 forms heteromeric channels with KCNQ4 in oocytes, using a dominant-negative mutant (KCNQ5-SYG) in which the first glycine of the ion selectivity filter of the pore is replaced with serine. KCNQ5-SYG eliminated currents generated in oocytes by coexpression with KCNQ4, consistent with KCNQ4/5 complex formation (*SI Appendix*, Fig. S7 *A* and *B*). Matrine and oxymatrine had negligible effects on KCNQ4/5, while aloperine effects were weaker than for homomeric KCNQ5, as were those of the mixture (*SI Appendix*, Fig. S7 *C–I* and Tables S7 and S8). Thus, while KCNQ3/5 and KCNQ4/5 currents are potentiated by aloperine, homomeric KCNQ5 is the most aloperine-sensitive KCNQ5-containing KCNQ channel.

Aloperine KCNQ-Dependently Relaxes Blood Vessels. Aloperine was reported recently to act as a vasodilator and hypotensive agent via an unknown mechanism (59). Here, based on our in vitro findings, we tested the ability of aloperine to relax ex vivo blood vessels, and whether this activity was KCNQ channel-dependent (Fig. 3*A*). Aloperine relaxed preconstricted third-order mesenteric arteries isolated from rat in a concentration-dependent manner, with $logEC_{50}$ of −4.72 ± 0.09 ($n = 12$; Fig. 3*B*). The presence of the KCNQ channel inhibitor linopirdine at both 3 and 50 μM inhibited the relaxations to aloperine, with the $logEC_{50}$ shifting to −4.21 ± 0.07 ($n = 6$; $P = 0.0012$) and −4.16 ± 0.04 ($n = 8$; $P = 0.0001$), respectively (Fig. 3*C*).

The linopirdine EC_{50} for KCNQ5 inhibition in *Xenopus* oocytes was previously quantified to be 51 μM (21), and here we found a similar value in oocytes, 46.1 ± 0.26 μM (*SI Appendix*, Fig. S8*A*). The greater linopirdine sensitivity of aloperine-activated artery relaxation we observed here, i.e., similar effects with 3 and 50 μM linopirdine, can be explained by 2 factors. First, linopirdine (10 μM) was previously found to completely eliminate the Kv current in A7r5 vascular smooth muscle cells, which express KCNQ5 but not KCNQ1–4, consistent with higher linopirdine potency in smooth muscle cells than in *Xenopus* oocytes (60), perhaps due to the known effects of the oocyte yolk in absorbing some drugs and lowering apparent sensitivity. Furthermore, 10 μM linopirdine previously inhibited the activating effects of the KCNQ channel activator BMS204352 on overexpressed KCNQ5 in HEK cells (61). Second, while we found in
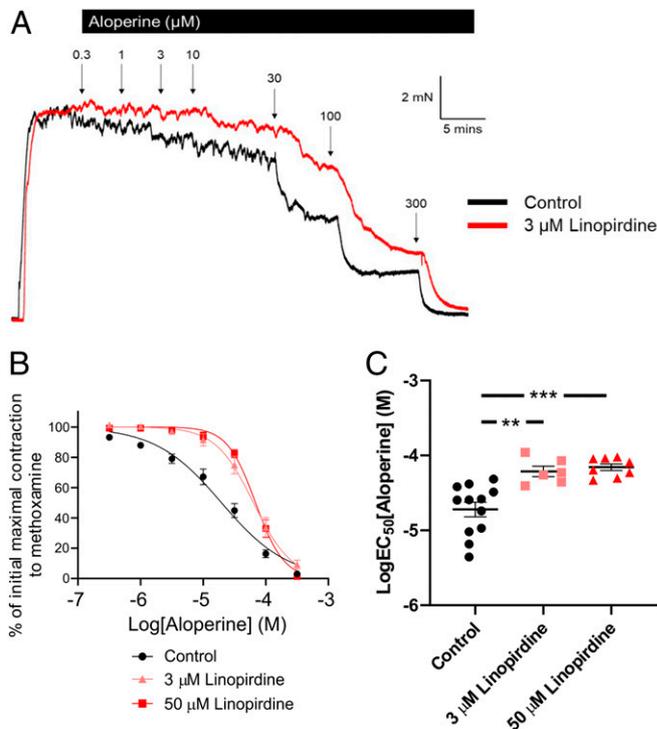

Fig. 3. Aloperine is a KCNQ-dependent vasorelaxant. All error bars indicate SEM. (*A*) Exemplar myography recordings of the tension changes initiated by aloperine (at concentrations indicated) alone (control) or with linopirdine (3 μM) in rat mesenteric artery precontracted with 10 μM methoxamine. (*B*) Dose response for relaxation of methoxamine precontracted rat mesenteric artery by aloperine alone (control) or in the presence of linopirdine as indicated ($n = 6$ to 12). (*C*) Scatter plot showing aloperine $logEC_{50}$ values for relaxation of methoxamine-precontracted rat mesenteric artery alone (control) or in the presence of linopirdine as indicated ($n = 6$ to 12; **$P < 0.01$ and ***$P < 0.001$).

oocytes that 50 μM linopirdine was more effective than 3 μM linopirdine at inhibiting aloperine potentiation of KCNQ5 and KCNQ4/5 outward currents, 3 μM linopirdine was nevertheless able to completely prevent the KCNQ5- and KCNQ4/5-dependent membrane hyperpolarizations induced by aloperine (10 μM; *SI Appendix*, Fig. S8 *B–K*). As vascular relaxation is dependent on the ability of baseline KCNQ channel activity at hyperpolarized voltages to further hyperpolarize the cell membrane (and not, per se, the size of more easily measurable outward currents at more depolarized voltages), these data provide further support that inhibition of aloperine-induced vascular relaxation by 3 μM linopirdine arises by preventing aloperine potentiation of KCNQ5 activity.

Aloperine Is a KCNQ5-Selective Activator. We next tested aloperine against homomers of the other retigabine-sensitive KCNQs, KCNQ2, KCNQ3* (KCNQ3-A315T, a variant that permits large enough currents for study of the homomeric channel) (62), KCNQ4, and the predominant neuronal isoform, heteromeric KCNQ2/3. None of these channels were sensitive to aloperine (100 μM; Fig. 4 *A–D*). Concentration response studies revealed that aloperine activates KCNQ5 with an EC_{50} of 390 ± 60 nM and that KCNQ2/3 and KCNQ2–4 homomers are relatively insensitive even to 1 mM aloperine (Fig. 4*E* and *SI Appendix*, Tables S3, S4, and S9–S11). The plant alkaloid aloperine is therefore a small-molecule channel opener that is selective for KCNQ5 versus KCNQ2-4.

The closest data point on our dose–response curve to our LC/MS-calculated minimum concentration of 47.1 nM aloperine in the 1% *S. flavescens* extract we used for electrophysiology is 100 nM aloperine, which increased KCNQ5 current ~4-fold at

Fig. 4. Aloperine is a potent and selective activator of KCNQ5 channels. All error bars indicate SEM. (*A*) Mean TEVC current traces showing effects of aloperine (100 μM) on KCNQ2/3 and homomeric KCNQ2, KCNQ3*, and KCNQ4 expressed in *Xenopus* oocytes (*n* = 5 to 10). (*B*) Mean tail current (*Left*) and mean normalized tail currents (G/Gmax; *Right*) versus prepulse voltage relationships for the traces as in *A* (*n* = 5 to 10). (*C*) Effects of aloperine (100 μM) on resting membrane potential (E_M) of unclamped oocytes expressing channels as in *A* (*n* = 5 to 10). n.s., not statistically significant (*P* > 0.05). (*D*) Voltage dependence of effects of aloperine (100 μM) on channels indicated (from traces as in *A*; *n* = 5 to 10). (*E*) Aloperine dose response calculated from fold increase in current at −60 mV for channels as indicated (*n* = 5 to 10).

−60 mV (Fig. 4*E*). This compares quite well with the ~6.5-fold increase we observed for KCNQ5 current at −60 mV with 1% *S. flavescens* extract (Fig. 3*J*), given the SE for data points at this voltage, the other compounds present in the extract that contribute minor effects, and the previously mentioned caveat that our concentration estimate is a conservative, minimum value due

to aloperine breakdown and an additional ambiguous peak that we could not definitively assign.

We also tested aloperine for activity against KCNQ1, the cardiac and epithelial isoform that lacks the S5 tryptophan required for activation by retigabine. Aloperine was able to moderately increase homomeric KCNQ1 activity (Fig. 5 *A* and *B*) and

Fig. 5. Aloperine weakly activates homomeric KCNQ1 but not KCNQ1-KCNE complexes. All error bars indicate SEM. (*A*) Mean TEVC current traces showing effects of aloperine (100 μM) on KCNQ1, KCNQ1/KCNE1, and KCNQ1/KCNE3 channels expressed in *Xenopus* oocytes (*n* = 5 to 6). (*B*) Mean tail current (*Left*) and mean normalized tail currents (G/Gmax; *Right*) versus prepulse voltage relationships for the traces as in *A* (*n* = 5 to 6). (*C*) Effects of aloperine (100 μM) on resting membrane potential (E_M) of unclamped oocytes expressing channels as in *A* (*n* = 5 to 6). n.s., not statistically significant (*P* > 0.05). (*D*) Voltage dependence of effects of aloperine (100 μM) on channels indicated (from traces as in *A*; *n* = 5 to 6). (*E*) Aloperine dose response calculated from fold increase in current at −60 mV for channels as indicated (*n* = 5 to 6).

induce a negative shift in E_M of KCNQ1-expressing oocytes (Fig. 5C). Aloperine exhibited much lower efficacy for KCNQ1 than we observed for KCNQ5 (Fig. 5 *D* and *E*) and a 12-fold lower potency (KCNQ1 EC_{50} = 4.6 ± 0.2 µM; Fig. 5*E* and *SI Appendix*, Table S12).

KCNQ1 is not thought to exist as a homomer in vivo; rather, it forms complexes with each of the KCNE β subunits in different tissues to create an array of functionally diverse channels (7). For example, KCNE1 slows KCNQ1 activation and positive-shifts its voltage dependence; the KCNQ1-KCNE1 channel is essential for ventricular myocyte repolarization and also hearing because of its role in regulating potassium secretion in the endolymph of the inner ear (63, 64). At the other extreme, KCNE3 locks open the KCNQ1 voltage sensor and pore, making the channel constitutively active; KCNQ1-KCNE3 is important for regulating cAMP-stimulated chloride secretion at the basolateral membrane of intestinal epithelia (65). Importantly, we found that KCNQ1-KCNE1 and KCNQ1-KCNE3 are essentially insensitive to aloperine (Fig. 5 *A–E* and *SI Appendix*, Tables S13 and S14). Therefore, while homomeric KCNQ1, which is not thought to exist in vivo, is moderately aloperine-sensitive, the physiologically relevant complexes KCNQ1 forms with KCNE1 and with KCNE3 are aloperine-insensitive.

Aloperine Binds to KCNQ5-R212. Retigabine and its derivatives, and also GABA and its metabolites and analogs, bind to KCNQ3-W265 and equivalent residues on one or more of the other neuronal KCNQs (16, 66). A shared feature of molecules that bind to the S5 tryptophan is a strong negative electrostatic surface potential close to a carbonyl group (20). Here, the (albeit modest) effects of aloperine on homomeric KCNQ1 suggested aloperine relies on a different residue(s) for binding, as KCNQ1 lacks the W265 equivalent. Furthermore, aloperine lacks the strong negative surface potential typical of W265 binding molecules and does not contain any carbonyl groups (Fig. 6*A*), also suggesting it might not require the S5 tryptophan for binding.

Recently, we found that the botanical compound mallotoxin, which activates KCNQ1–5 homomers, binds close to and requires for activity an arginine between the S4–5 linker and S4 (67, 68) (R213 in KCNQ2, R242 in KCNQ3, R212 in KCNQ5), mutation of which (in KCNQ2) is associated with benign familial neonatal convulsions (69). Here, we performed in silico docking studies using a chimeric model constructed from the cryo-EM–derived KCNQ1 structure (70) (Fig. 6*B*) with KCNQ3/5 residues important for retigabine binding introduced. This predicted that, like mallotoxin, aloperine binds close to the arginine at the foot of the voltage sensor (R212 in KCNQ5), which lies across from and deeper than W235 in KCNQ5 S5 (Fig. 6 *C* and *D*). We tested this prediction using KCNQ5-R212A channels expressed in oocytes, which behave at baseline similar to wild-type KCNQ5. The R212A mutation eliminated KCNQ5 aloperine sensitivity
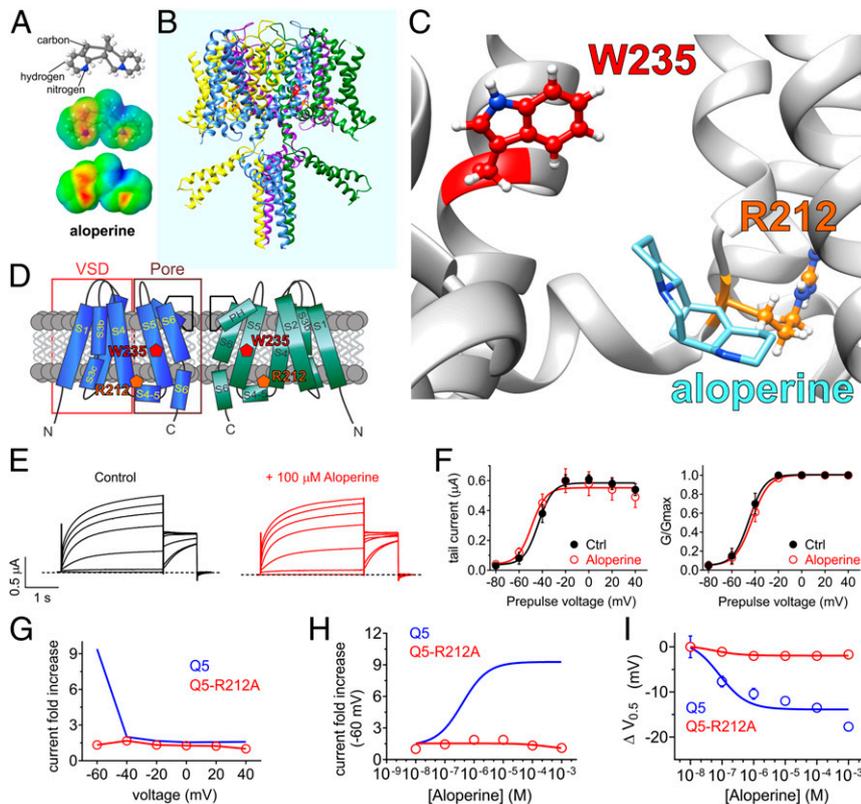

Fig. 6. Aloperine activation of KCNQ5 requires KCNQ5-R212. All error bars indicate SEM. (*A*) Aloperine chemical structure (*Upper* and *Center*) and electrostatic surface potentials (red, electron-dense; blue, electron-poor; green, neutral; *Lower* and *Center*) calculated and plotted using Jmol. (*B*) Chimeric KCNQ1/KCNQ5 structural model (orange, KCNQ5-R212; red, KCNQ5-W235). (*C*) View of the aloperine binding site in KCNQ5 predicted by SwissDock (and see Fig. 7*A*). (*D*) Topological representation of KCNQ5 showing 2 of the 4 subunits, without domain swapping for clarity. Pentagons indicate approximate position of KCNQ5-R212 (orange) and KCNQ5-W235 (red). VSD, voltage sensing domain. (*E*) Mean TEVC current traces showing effects of aloperine (100 µM) on KCNQ5-R212 expressed in *Xenopus* oocytes (*n* = 5). (*F*) Mean tail current (*Left*) and mean normalized tail currents (G/Gmax; *Right*) versus prepulse voltage relationships for the traces as in *E* (*n* = 5). (*G*) Voltage dependence of effects of aloperine (100 µM) on channels indicated (from traces as in *E* and Fig. 2*C*; *n* = 5). (*H*) Aloperine dose response calculated from fold increase in current at −60 mV for channels as indicated (*n* = 5). (*I*) Aloperine dose response calculated from $\Delta V_{0.5activation}$ for channels as indicated (*n* = 5).

(Fig. 6 *E–G*), even at concentrations as high as 1 mM (Fig. 6 *H* and *I* and *SI Appendix*, Table S15). Together with the docking simulations, the findings suggest that aloperine requires KCNQ5-R212 for binding and/or channel activation.

Aloperine KCNQ Isoform Selectivity Is Independent of Binding. Aloperine docked closest to R212 and required it for activation (Fig. 6). However, an examination of all of the predicted aloperine binding poses close to R212 (Fig. 7 *A*, *Left*) suggests that aloperine could impinge upon the KCNQ GABA binding site, which we previously found (66) to be in a similar pocket to that predicted for aloperine but reliant on the S5 tryptophan (W235 in KCNQ5). Even the more centrally placed of the various aloperine in silico binding poses, when plotted as a spacefill model, suggests aloperine could impinge upon small-molecule binding to W235 (Fig. 7 *A*, *Right*). By the same token, this suggests that GABA, which requires the S5 W for binding and activation (66), might also be influenced by R212. We tested this hypothesis directly, using a tritiated GABA binding assay as

before (66), and found that mutation of KCNQ5-R212 to alanine reduced binding >3-fold (Fig. 7*B*).

This result further refines the KCNQ GABA binding site, and also facilitated use of competition with tritiated GABA to analyze aloperine binding to different KCNQ isoforms. GABA did not bind to KCNQ1, but bound to KCNQ5, recapitulating our previous findings (66). Aloperine (100 μM) reduced GABA (1 μM) binding, with no additional competition produced by 1 mM aloperine (Fig. 7 *B* and *C*). The GABA EC_{50} for KCNQ5 activation is 60 nM (66), compared to 390 nM for aloperine (Fig. 4*E*). The data, together with docking and mutagenesis data from the present study and our previous study (66), are consistent with GABA and aloperine occupying somewhat different positions in the same binding pocket, as a 1,000-fold excess of aloperine diminished but did not completely eliminate GABA binding.

The isoform selectivity of aloperine effects, despite the conservation of the KCNQ5-R212 position within the KCNQs (Fig. 7*D*), suggests that selectivity must be provided by functional effects downstream of binding, rather than by binding selectivity. Indeed, we previously found that GABA binds to KCNQ2–5 but only activates KCNQ3 and KCNQ5 (66). We again utilized competition with tritiated GABA to test this hypothesis, and found that aloperine (100 μM) is able to diminish binding of tritiated GABA (1 μM) to homomeric KCNQ2, 3, and 4 channels, despite aloperine not activating these isoforms (Fig. 7*E*). Our findings suggest that, like GABA, aloperine binds to multiple KCNQ isoforms because of a conserved binding site (involving R212 and equivalents, in the case of aloperine), and that there is isoform dependence of the functional effects of binding arising from yet-to-be-determined sequence elements elsewhere in the channel.
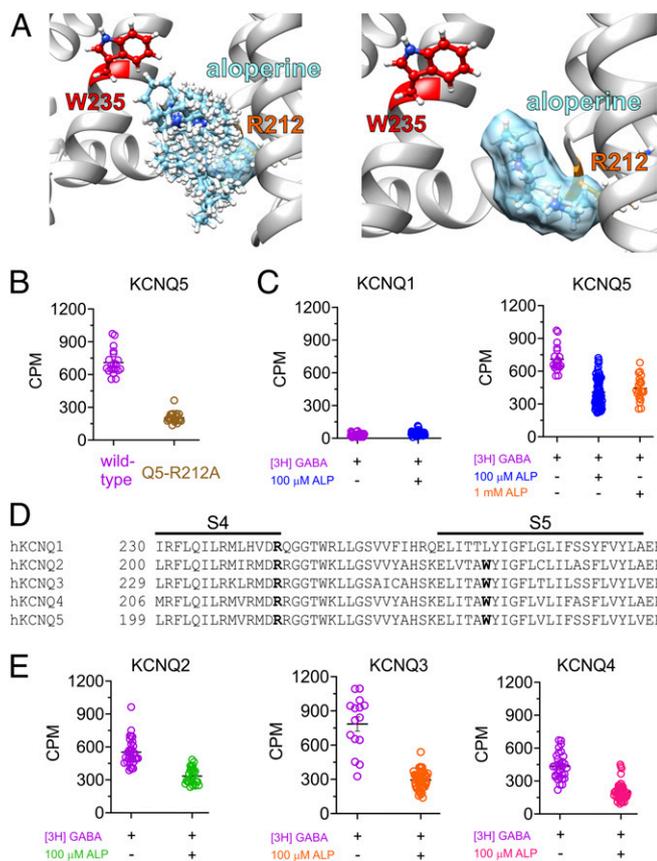
Discussion
Folk medicines are in daily widespread use across the globe. In addition, many of the herbs and other plants in daily use as foods or food flavorings have historically been utilized for purported medicinal purposes; in some cases, this use dates back millennia. Herbs such as thyme, oregano, and basil are in daily use by hundreds of millions of people.

In the present study, we report the striking finding that a range of traditional hypotensive botanical medicines from diverse plant clades and families, usage of each of which has arisen in often highly distinct, temporally and geographically separated human cultures, share the common property of KCNQ5 potassium channel activation. This is particularly striking because the same plant extracts are inactive against KCNQ2/3, the predominant neuronal KCNQ channel type. KCNQ5, together with KCNQ1 and KCNQ4, is expressed in vascular smooth muscle, where its activation relaxes blood vessels (26), making KCNQ5 activation a logical mechanism for at least part of the hypotensive actions of these folk medicines. Another notable feature of the discovery is that KCNQ5-selective agonists, which exhibit negligible activity for KCNQ2–4 isoforms, have apparently eluded conventional screening methods utilizing chemical libraries.

With respect to isolating specific compounds, we focused on *S. flavescens*, which produced both voltage-dependent and voltage-independent KCNQ5 opening and was highly selective for KCNQ5 over KCNQ2/3 (Fig. 1 and *SI Appendix*, Fig. S3). This enabled us to uncover aloperine as a first-in-class preferential KCNQ5 opener. It is anticipated that the other hypotensive plant extracts we discovered to activate KCNQ5 harbor a treasure trove of KCNQ5-selective activators. These plants have been used for millennia with the intention of treating one or more disorders, as documented in texts dating back as many as 5,000 y (1–3). In addition, traces of chamomile flower, which we found to strongly activate KCNQ5 even at −80 mV (*SI Appendix*, Fig. S2), have been detected in the dental calculus of *Homo neanderthalensis* skulls dated at 48,000 y old. In the same study, traces

Fig. 7. Aloperine KCNQ isoform selectivity is independent of binding selectivity. All error bars indicate SEM. (*A*, *Left*) Superimposition of all of the aloperine binding poses in the neurotransmitter binding pocket of KCNQ5 predicted by SwissDock. (*A*, *Right*) Aloperine spacefill model in the central binding pose of all of the superimposed binding poses (*Left*). (*B*) Comparison of tritiated GABA binding (for these and all following panels, bound GABA activity is expressed in counts per minute [cpm] to wild-type and R212A KCNQ5 expressed in oocytes; n = 20 to 22). (*C*) Effects of aloperine on tritiated GABA binding to KCNQ5 (n = 20 to 74); KCNQ1 (n = 37 to 41) was used as a negative control for GABA binding. (*D*) Amino acid sequence alignment of the S4 to S5 regions of human KCNQ1–5. KCNQ5-R212 and KCNQ5-W235 (and equivalents) are shown in bold. (*E*) Effects of aloperine on tritiated GABA binding to KCNQ2 (n = 33 to 57), KCNQ3 (n = 15 to 48), and KCNQ4 (n = 31 to 55).

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of willow (which contains the painkiller salicylic acid or aspirin) were found exclusively in the dental calculus of an *H. neanderthalensis* skull bearing a dental abscess, suggesting that Neanderthals were self-medicating (5, 6). Because of its bitterness, chamomile is not considered food, also suggesting this may have been consumed medicinally by Neanderthals, although it seems highly unlikely it would have been consumed for its hypotensive properties. Chamomile and fennel seeds were also found in 800,000-y-old strata associated with Paleolithic hominin activity at the Gesher Benot Ya'aqov site in Israel (4). We found fennel seed extract to be among the most efficacious KCNQ5 activators (Fig. 1 *D* and *E*). While fennel seed discovery at Gesher Benot Ya'aqov may have been associated with ingestion of fennel as a food source, the presence of chamomile (not considered a food) again points to the possibility that early hominins such as *Homo erectus* were self-medicating (4). Either way, ingestion of chamomile and fennel seed may well have been activating the KCNQ5 channels of early hominins, depending on whether or not their preparation techniques, including the possibility of heating, preserved this activity; controlled use of fire by early human species may date back as far as 1.5 million years (71).

KCNQ5-selective openers based on aloperine, related molecules, or other compounds we have yet to uncover in the other hypotensive extracts with KCNQ5 activity may eventually be developed for clinical use for hypertension or, for example, KCNQ5 loss-of-function encephalopathies (32, 33). Aloperine has proven hypotensive properties (59), and we show here that its vasorelaxant effects are KCNQ-dependent, being inhibited by the KCNQ channel inhibitor linopirdine. We also demonstrate that aloperine is highly selective for KCNQ5 over other KCNQ isoforms, with modest effects on homomeric KCNQ1. We do not consider the effects on KCNQ1 a hurdle in terms of potential future usage as a diagnostic (or even clinical) KCNQ5 opener in biological systems, as KCNQ1 is not thought to exist as a homomer in vivo, but is always complexed with one or more of the KCNE β subunits that dictate its functional properties (7). However, mechanistically, the KCNQ1 effect indicates that aloperine is not perfectly KCNQ5-selective. This property may be achievable by examining other natural aloperine derivatives from *Sophora* species and others, or synthetic derivatives that have already been reported (72). In the wider sense, aloperine also interacts with other, nonchannel proteins (72), which may also be beneficial clinically, but could possibly limit its usefulness as a pharmacological tool in determining precise physiological roles of KCNQ5 in vivo.

There are noticeable differences in the KCNQ5-activating efficacy between the various 1% plant extracts (Fig. 1 and *SI Appendix*, Fig. S2); fennel seed and lavender exert greater shifts in KCNQ5 $V_{0.5act}$, but others including thyme, oregano, and Vietnamese *Sophora* root are less efficacious. There are at least 4 possible explanations for this. First, either the active compounds in these plant extracts exhibit different potency or efficacy than those in *S. flavescens*, or second, the active compounds are in substantively different concentrations in the various extracts. Third, and most likely given the diversity of plants analyzed, the variance arises from a combination of both these effects. Fourth, for consistency, we used the 80% methanol extraction technique for all 15 plants studied. However, this may not be the optimal extraction technique for all of the plants,

depending on their consistency and chemical composition, and extraction technique is well known to influence the efficacy of herbal medicines, as one might expect (37, 54). We anticipate that, with extensive future analyses, one or more of the other hypotensive botanical extracts we found to activate KCNQ5 will yield superior and perhaps more specific KCNQ5-activating small molecules than aloperine, with possible clinical implications.

Materials and Methods

Full details of the study methods are provided in the *SI Appendix*.

In Vitro Functional and Chemical Analysis of Plant Extracts and Compounds. We functionally screened 1% plant extracts or individual compounds from *S. flavescens* using TEVC of *X. laevis* oocytes expressing the KCNQ and KCNE subunits indicated using an OC-725C amplifier (Warner Instruments) and pClamp10 software (Molecular Devices). Bath solution was (in mM) 96 NaCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 Hepes (pH 7.6). We recorded currents in response to voltage pulses between −120 or −80 mV and +40 mV at 20-mV intervals from a holding potential of −80 mV to yield current–voltage relationships and current magnitude and for quantifying activation rate. We analyzed *S. flavescens* extract with GC/MS and LC/MS to detect and quantify aloperine content. For GABA binding competition assays, we incubated oocytes with 1 μM γ-[2,3-³H(N)]-aminobutyric acid (³H-GABA; Perkin–Elmer) at 25 to 40 Ci/mMol specific activity for 30 min, alone or with aloperine (100 μM or 1 mM), washed the oocytes 4 times, lysed in SDS, transferred to Cytoscint scintillation mixture fluid (MP Biomedicals), and then quantified radioactivity with a Beckman Coulter LS6500 liquid scintillation counter.

Mesenteric Artery Myography. Rat studies were performed according to the *Guide for the Care and Use of Laboratory Animals* (73) and approved by the National Ethics Committee, Denmark. Male Wistar rats (13 to 14 wk old) were killed by cervical dislocation before the intestines were removed and third-order mesenteric arteries dissected in ice-cold physiological saline solution. Changes in tension of arterial segments were recorded continuously on a wire myograph (Danish Myo Technology). In the absence or presence of linopirdine (3 or 50 μM; Sigma), artery segments were precontracted with 10 μM methoxamine before application of aloperine (Sigma).

Chemical Structures and Silico Docking. Chemical structures were plotted using Jmol: http://jmol.sourceforge.net/. For docking, we used the *X. laevis* KCNQ1 cryoEM structure (70) altered to incorporate KCNQ3/KCNQ5 residues known to be important for retigabine and ML-213 binding, and their immediate neighbors, followed by energy minimization using the GROMOS 43B1 force field (74), in DeepView (75). We then performed unguided docking of aloperine using SwissDock (76) with CHARMM forcefields (77).

Statistical Analysis. All values are expressed as mean ± SEM. One-way ANOVA was applied for all other tests; if multiple comparisons were performed, a post hoc Tukey's HSD test or Šidák's multiple comparisons test was performed following ANOVA. All *P* values were 2-sided. Statistical significance was defined as $P < 0.05$.

Data and Materials Availability. All datasets are provided in the main text and *SI Appendix*; materials are available upon reasonable request.

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