Synthetic resin acid derivatives selectively open the hKV7.2/7.3 channel and prevent epileptic seizures

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Abstract

Objective: About one third of all patients with epilepsy have pharmacoresistant seizures. Thus there is a need for better pharmacological treatments. The human voltage-gated potassium (hKV) channel hKV7.2/7.3 is a validated antiseizure target for compounds that activate this channel. In a previous study we have shown that resin acid derivatives can activate the hKV7.2/7.3 channel. In this study we investigated if these channel activators have the potential to be developed into a new type of antiseizure drug. Thus we examined their structure-activity relationships and the site of action on the hKV7.2/7.3 channel, if they have unwanted cardiac and cardiovascular effects, and their potential antiseizure effect.

Methods: Ion channels were expressed in Xenopus oocytes or mammalian cell lines and explored with two-electrode voltage-clamp or automated patch-clamp techniques. Unwanted vascular side effects were investigated with isometric tension recordings. Antiseizure activity was studied in an electrophysiological zebrafish-larvae model.

Results: Fourteen resin acid derivatives were tested on hKV7.2/7.3. The most efficient channel activators were halogenated and had a permanently negatively charged sulfonyl group. The compounds did not bind to the sites of other hKV7.2/7.3 channel activators, retigabine, or ICA-069673. Instead, they interacted with the most extracellular gating charge of the S4 voltage-sensing helix, and the effects are consistent with an electrostatic mechanism. The compounds altered the voltage dependence of hKV7.4, but in contrast to retigabine, there were no effects on the maximum conductance. Consistent with these data, the compounds had less smooth muscle-relaxing effect than retigabine. The compounds had almost no effect on the voltage dependence of hKV11.1, hNaV1.5, or hCaV1.2, or on the amplitude of hKV11.1. Finally, several resin acid derivatives had clear antiseizure effects in a zebrafish-larvae model.

Significance: The described resin acid derivatives hold promise for new antiseizure medications, with reduced risk for adverse effects compared with retigabine.

Keywords
epilepsy, excitability, potassium channel opener
1 | INTRODUCTION

About 65 million people worldwide have epilepsy, and of these, approximately one third have pharmaco-resistant disease.\(^1,2\) Thus there is a great need for better treatment. Epilepsy is characterized by recurrent seizures, and one obvious target for antiseizure drugs is the neuronal excitability-regulating M-current.\(^3\) The M-current is generated by a group of tetrameric voltage-gated potassium (K\(_V\)) channels, K\(_V\)7.2–7.5, encoded by the KCNQ2-5 genes;\(^4\) in most cases, the M-channel consists of a mix of K\(_V\)7.2 and K\(_V\)7.3 subunits (K\(_V\)7.2/7.3).\(^5\) Each subunit has six transmembrane segments, where transmembrane segments 1 to 4 (S1–S4) form peripheral voltage sensor domains (VSDs), and transmembrane segments 5 to 6 (S5–S6) form the central ion-conducting pore domain. The human (h) K\(_V\)7.2/7.3 channel is partly open at resting potential and does not inactivate. Further opening of this channel hyperpolarizes the membrane and increases the action potential afterhyperpolarization period.\(^6\) The importance of this current in controlling neuronal activity is highlighted by mutations that reduce the M-current, thereby causing epilepsy.\(^7,8\) Thus opening the hK\(_V\)7.2/7.3 channel is a rational pharmacological strategy to prevent epileptic seizures.

Retigabine (RTG), approved as an add-on treatment for drug-resistant partial-onset seizures in 2011,\(^9\) exerts its antiseizure effect by stabilizing the open conformation of the hK\(_V\)7.2/7.3 channel.\(^10\) It binds to the central pore domain of the channel, specifically to a tryptophan in S5, which is conserved among the hK\(_V\)7.2–7.5 channels (green residue in Figure 1A; molecular structure in Figure 1B); therefore, RTG is not selective for hK\(_V\)7.2/7.3 but also activates the hK\(_V\)7.4 and hK\(_V\)7.5 channels.\(^11\) RTG was associated with several side effects, which were possibly due to activation of Kv7.4 and Kv7.5 channels in various organs.\(^12\) In addition, RTG caused a blue-gray mucocutaneous discoloration, which depends on photo instability of a metabolite.\(^13\) Due to the side effects, the use of RTG was limited and it was withdrawn from the market in 2017.\(^14\) Despite these drawbacks, RTG validated the hK\(_V\)7.2/7.3 channel as an antiseizure target, thereby supporting the development of new hK\(_V\)7.2/7.3 channel activators as potential antiseizure drugs. However, to minimize the risk of adverse effects, new activators should be more selective to the hK\(_V\)7.2/7.3 channel. To avoid activating hK\(_V\)7.4 and hK\(_V\)7.5 channels, it is reasonable to target an alternative to the conserved RTG site, potentially on the VSDs.

Several potential sites for activators have been identified in the VSDs of hK\(_V\)7.2/7.3 channels. One is in a cleft between S1, S2, and S4, which is directly accessible from the extracellular solution.\(^14\) However, this site is not selective, as it is also found in other hK\(_V\) channels as well as in hNa\(_V\) channels.\(^15,16\) Another site is located on S3. Benzamides, like ICA-069673 (from hereon called ICA-73, Figure 1C), stabilize the activated conformation of the voltage sensor by interacting with residues in S3; both the extracellular and intracellular (F168 of hK\(_V\)7.2; pink residue in Figure 1A) ends of S3 are important for the interaction, but details for its binding site are still missing.\(^17,18\) Another hK\(_V\)-channel activator, ztz240, is suggested to bind to the gating charge transfer center of the hK\(_V\)7.2 channel, making interactions with residues in S3 (F137 and D172), and the gating charges R4 and R5 in S4 (R207 and R210 respectively).\(^19\)

Another strategy to open the channel is to let a negatively charged compound bind on the lipid-facing side of the channel, close to the extracellular end of the positively charged voltage sensor S4, to electrostatically attract gating charges of S4 (blue residues in Figure 1A). We have suggested that resin acids and their chemical derivatives act via this “lipoelectric” mechanism\(^20\) by binding in the lipid-facing groove between S3 and S4 as demonstrated in the Shaker K\(_V\) channel from the fruit fly Drosophila melanogaster.\(^21\) From this binding site, the resin acid compounds electrostatically facilitate the last rotational movement of the voltage sensor toward the activated state, thereby pulling the gate open.\(^21,22\) Using the Shaker K\(_V\) channel mutated to be extra sensitive for compounds acting by the lipoelectric mechanism,\(^24\) we have investigated the opening effect of 145 naturally occurring resin acids and synthesized resin acid derivatives, which has provided information about the structure-activity relationship for this group of compounds.\(^21,23,25\) In a recent study, we described that some resin acid derivatives also activated the hK\(_V\)7.2/7.3 channel.\(^23\) By replacing the carboxyl group of a naturally occurring resin acid (ie, dehydroabietic acid [DHAA]; Figure 3A) with a sulfonyl group at the end of a stalk of two atoms, thereby separating the negative charge from the lipophilic three-ring structure, we generated the hK\(_V\)7.2/7.3 channel activator Wu161 (Figure 1D).\(^23\)

Key Points

- Resin acids and their derivatives open the hK\(_V\)7.2/7.3 channel by an electrostatic interaction with gating charges in the voltage sensor S4.
- The derivative Wu200 is a potent and efficient activator of the hK\(_V\)7.2/7.3 channel.
- In contrast to retigabine, resin acid derivatives do not increase the maximum conductance of hK\(_V\)7.4, and Wu200 has a smaller vasorelaxant effect than retigabine.
- The resin acid derivatives have almost no effects on the voltage dependence of hK\(_V\)11.1, hNa\(_V\)1.5, or hCa\(_V\)1.2.
- Several resin acid derivatives have antiseizure effects in a zebrafish model.
This suggests that resin acids can form a scaffold for new antiseizure drugs.

In the present study, we investigated if the resin acid derivatives activate the hKV7.2/7.3 channel via the lipoelectric mechanism. To determine the site of action on the hKV7.2/7.3 channel, we used Wu161 as a prototypical resin acid derivative. By further chemical modification, we aimed to improve the potency and efficacy of Wu161. To predict unwanted cardiac and vascular effects, we examined the effects of the hKV7.2/7.3 activators on a selection of ion channels and isolated arteries and compared the effect to that of RTG. Finally, we explored if our most effective hKV7.2/7.3 channel activators prevent seizures in an epilepsy model.

2 | RESULTS

2.1 | Wu161 activates hKV7.2, hKV7.3, and hKV7.2/7.3

To explore the site and mechanism of action of the resin acid derivatives, we measured ion currents by the two-electrode voltage clamp technique. First, we explored whether the compounds were selective for heteromeric (hKV7.2/7.3) or homomeric (hKV7.2 and hKV7.3) channels expressed in oocytes from *Xenopus laevis*. For the heteromeric hKV7.2/7.3 channel, 30 µM Wu161 (Figure 1D) increased the current by a factor of 6.0 (black arrow).
OTTOSSON et al.

The relative conductance-versus-voltage, \( G(V) \), curve was obtained from the instantaneous current at \(-30\ \text{mV}\) following different voltage steps from \(-120\ \text{mV}\) to \(+30\ \text{mV}\). Application of 30 \( \mu \text{M} \) Wu161 shifted this curve in a negative direction along the voltage axis and increased the maximum conductance \( (G_{\text{MAX}}) \) shift (Figure 1F). If the desired therapeutic action is to open the hK\(_{\text{V}}\)7.2/7.3 channel at resting potential (to hyperpolarize the resting neuron, and thereby to dampen neuronal firing) we want to know what a pharmacological compound is doing to the channel at \(-60\) to \(-70\ \text{mV}\). At these voltages, the channel opening is almost completely caused by the \( G(V) \) shift (dashed lines in Figure 1F). Therefore, we focused mainly on the \( G(V) \) shift in the present investigation, but as will be shown below, for other channels where the compounds cause other effects, the increase in \( G_{\text{MAX}} \) can have an advantageous or a disadvantageous effect.

The hK\(_{\text{V}}\)7.3 homomer currents are very small. To facilitate their study, a point mutation A315T was introduced to increase the size of the current (hK\(_{\text{V}}\)7.3[A315T], hereinafter referred to as hK\(_{\text{V}}\)7.3*). Wu161 (30 \( \mu \text{M} \)) activated both hK\(_{\text{V}}\)7.2 (Figure 1G) and hK\(_{\text{V}}\)7.3* (Figure 1H), with no difference of induced shift of \( G(V) \) or increase of \( G_{\text{MAX}} \) (Figure 1F-H). The \( E_{\text{C50}} \) of Wu161 did not differ between the channels (Figure 1I). However, the maximally induced shift \( (\Delta V_{\text{MAX}}) \) differs, largest for hK\(_{\text{V}}\)7.2 and least for hK\(_{\text{V}}\)7.2/7.3 (Figure 1I).

To explore if the effect of Wu161 depends on the expression system, we also investigated Wu161 on the hK\(_{\text{V}}\)7.2/7.3 channel expressed in a human cell line (HEK cells). Using an automated patch-clamp technique, we found no difference between the results of the two cell types and the two techniques (Figure 1I). Consequently, this suggests that data for the voltage dependence from the two methods are comparable.

### 2.2 Wu161 interacts with the voltage sensor S4 and not with the retigabine or benzamide sites

The clinically validated antiseizure drug RTG (Figure 1B) activates the hK\(_{\text{V}}\)7.2/7.3 channel, mainly by shifting the \( G(V) \) curve in a negative direction along the voltage axis (Figure 2A). RTG binds to a critical tryptophan found in both hK\(_{\text{V}}\)7.2 (W236, Figure 1A) and hK\(_{\text{V}}\)7.3 (W265). Mutations of these tryptophans to leucines (hK\(_{\text{V}}\)7.2[W236L]/7.3[W265L]) render the channel insensitive to RTG (Figure 2B,C). In contrast, the effects of Wu161 on hK\(_{\text{V}}\)7.2/7.3 are not sensitive to these mutations (Figure 2B,C), suggesting that Wu161 does not bind to the RTG site.

The benzamide ICA-73 (Figure 1C) is a potent activator of hK\(_{\text{V}}\)7.2 that shifts the \( G(V) \) in a negative direction along the voltage axis. At 30 \( \mu \text{M} \) ICA-73, the channel did not close completely, even at \(-120\ \text{mV}\) (Figure 2D), as described in the literature. Several residues along S3 are important for the effects of benzamides, but the hK\(_{\text{V}}\)7.2 mutation F168L alone (in the S2–S3 linker) completely removes the effect of ICA-73 (Figure 2E,F). The effect of Wu161 is not affected by the hK\(_{\text{V}}\)7.2 mutation F168L (Figure 2E,F), suggesting that Wu161 opens the channel by acting on amino acid residues other than those targeted by ICA-73.

Previous studies on the Shaker K\(_{\text{V}}\) channel suggest that the lipophilic compound Wu161 binds close to the lipid bilayer and acts electrostatically on the voltage sensor S4, a mechanism we refer to as the lipoelectric mechanism; the valence of the charge of the compound and the valence of the charge of the voltage sensor are crucial for the effect.

To investigate if the lipoelectric mechanism underlies the effect of Wu161 (Figure 1D) on hK\(_{\text{V}}\)7.2, we explored the effect on hK\(_{\text{V}}\)7.2[R198Q] (ie, hK\(_{\text{V}}\)7.2[R1Q]). The charge-neutralization mutation reduced the induced \( G(V) \) shift by 50\% (Figure 2H,I), suggesting that at least part of the \( G(V) \)-shifting effect of Wu161 is caused by an electrostatic interaction with R1 in the voltage sensor S4. Of interest, the mutation R198Q can cause epilepsy, and R198 has been implicated in the effects of fenamates.

Taken together, these data suggest that Wu161, and possibly other resin acid derivatives, open the hK\(_{\text{V}}\)7.2/7.3 channel by interacting with S4 electrostatically, at a site separate both from that for RTG and benzamides.

### 2.3 Chemical groups important for channel activation

In the next step we sought to explore which chemical groups of the resin acid compounds are important for the effect by investigating one naturally occurring resin acid (DHAA, Figure 3A) and 13 resin acid derivatives (Figure 3A,C). The compounds have an acidic group attached to a three-ringed hydrophobic part. We explored two types of acidic groups: (1) a carboxyl group directly attached to carbon 4 (C4, see DHAA in Figure 3A for naming of positions), and (2) a sulfonyl group attached to a stalk composed of two extra atoms (Figure 3C). The sulfonyl group compounds are permanently negatively charged and reach their full effect as activators at physiological pH, whereas the carboxyl group compounds require a basic pH to reach their full effect as activators. To ensure that the carboxyl group is fully negatively charged, to be able to compare results between the two groups of compounds, we performed the experiments with the carboxyl group compounds at pH 9. pH 9 has only small effects on the hK\(_{\text{V}}\)7.2/7.3 channel's voltage dependence; at pH 7.4 the midpoint of the \( G(V) \) curve (\( V_{\text{50}} \); Equation 2) is \(-41.4 \pm 0.5\) (\( n = 141 \)), whereas at pH 9.0 it is \(-43.0 \pm 0.8\) (\( n = 41 \)), a difference of only 1.6 mV. The sulfonyl group compounds were tested at pH 7.4.
For the carboxyl group compounds, we found that (Figure 3B): (1) Halogenation of C12 increased the $G(V)$ shift from $-5.3 \pm 1.1$ mV (DHAA) to $-11.7 \pm 0.6$ mV (Wu13), $-17.6 \pm 0.9$ mV (Wu24), and $-19.3 \pm 1.4$ mV (Wu60) respectively. (2) The identity of the halogenation was crucial; the larger halogen, the larger the effect. (3)
A methyloxime side chain attached to C7 did not increase the effect (c.f. Wu36 vs DHAA; and Wu32 vs Wu24). This contrasts with what we have reported for the 3R Shaker KV channel, suggesting a different microenvironment around the bound compound.25 (4) Moving the Cl from C12 (Wu13) to C14 (Wu14) almost doubled the shift from −11.6 ± 0.6 to −22.9 ± 1.1 mV. (5) Combination of Cl at both C12 and C14 (Wu27) had no additional effect. (6) Substituting the isopropyl group at C13 with a Cl (Wu50) reduced the effect, which is in sharp contrast to the effect on the 3R Shaker KV channel.25

For the sulfonyl group compounds we found that: (1) The effect does not depend on the atomic identity of the stalk (Figure 3D). (2) For the compounds with an unhalogenated lipophilic part, the sulfonyl group on a stalk of two atoms (with N in the stalk) increased the shift from −5.3 ± 1.1 to −14.5 ± 0.7 mV (Figure 3E). (3) For the monohalogenated compounds, the exchange from a carboxyl group to a sulfonyl

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**FIGURE 3** Structural modifications of the resin acid derivatives affects channel activation. A, Molecular structures of dehydroabietic acid (DHAA, including numbering of some carbon atoms) and investigated resin acid derivatives with a carboxyl group. B, G(V) shifts (ΔV50) (hKV7.2/7.3 expressed in Xenopus oocytes) at pH 9 by 30 µM of indicated compound. Data = mean ± SEM. Color coding according to halogenation as indicated. G(V) shifts: DHAA: −5.3 ± 1.1 mV (n = 5); Wu13: −11.6 ± 0.6 mV (n = 6); Wu24: −17.6 ± 0.9 mV (n = 4); Wu60: −19.3 ± 1.4 mV (n = 6); Wu32: −13.6 ± 1.5 mV (n = 4); Wu36: −4.3 ± 0.5 mV (n = 5); Wu14: −22.9 ± 1.1 mV (n = 4); Wu27: −20.9 ± 2.0 mV (n = 5); Wu50: −13.3 ± 0.8 mV (n = 4). One-way ANOVA was used with Sidak's multiple comparison test. For comparison with DHAA (dotted line), the significance is given above each bar. ns, p > .05; *, p < .05; **, p < .01; ***, p < .001; ****, p < .0001. C, Molecular structures of investigated resin acid derivatives with a sulfonyl group. D, Concentration-response for ΔV50 (hKV7.2/7.3 expressed in Xenopus oocytes) at pH 7.4 for Wu109 and Wu161 as indicated. Data = mean ± SEM. Equation 3 for Wu109: ΔVMAX = −38.8 ± 4.5 mV, EC50 = 51.1 ± 12.6 µM, (n = 3–4). Equation 3 for Wu161: ΔVMAX = −31.7 ± 3.0 mV, EC50 = 22.7 ± 5.8 µM, (n = 4–8). E, G(V) shifts (ΔV50) (hKV7.2/7.3 expressed in Xenopus oocytes) at pH 9.0 (30 µM DHAA, Wu24, and Wu14 [for data see B]) or at pH 7.4 (Wu109: −14.5 ± 0.7 mV (n = 6); Wu199: −22.6 ± 2.6 mV (n = 4); Wu200: −27.6 ± 1.4 mV (n = 4). Data = mean ± SEM. Color coded as in B. ANOVA with Sidak's multiple comparisons test was used. ns, p > .05; *, p < .05; **, p < .01; ***, p < .001; ****, p < .0001. F, Concentration-response for ΔV50 (hKV7.2/7.3 expressed in Xenopus oocytes) at pH 7.4 for Wu109, Wu199, and Wu200 as indicated. Data = mean ± SEM. Equation 3 for Wu109: ΔVMAX = −31.9 ± 1.8 mV, EC50 = 12.3 ± 2.2 µM, (n = 3–8). Equation 3 for Wu200: ΔVMAX = −37.2 ± 1.2 mV, EC50 = 11.7 ± 1.3 µM, (n = 4–7). For Wu109, see D. (G) G(V) shifts (ΔV50) (hKV7.2/7.3 expressed in Xenopus oocytes) at pH 7.4 by 30 µM of indicated compound. Data = mean ± SEM. Resin acid derivatives are color coded as in B. Data for RTG from Figure 2C. Data for Wu161 from Figure 1E. Data for Wu109, Wu199, and Wu200 from E. Wu181: −34.1 ± 3.1 mV (n = 5). ANOVA with Dunnett's multiple comparison test was used for comparison with RTG (dashed line). ns, p > .05; **, p < .01; ***, p < .001; ****, p < .0001.
**FIGURE 4** Retigabine (RTG) and resin acid derivatives open the Kv7.4 channel and induce linopirdine-sensitive relaxation. A. Currents through hKv7.4 (expressed in *Xenopus* oocytes) in control and RTG as indicated. Holding voltage = −100 mV; voltage step 1 (3 s) = −20 mV; voltage step 2 (2 s) = −30 mV (tail currents). The instantaneous current at step 2 was increased by a factor of 10 (black arrow). B. G(V) curves for recordings as in A. Data = mean ± SEM. Equation 2: V_{50} = −21.1 ± 1.0 mV, −30.9 ± 1.2 mV, ΔV_{50} = −9.8 ± 0.5 mV; G_{MAX} increased by a factor of 6.1 ± 0.2 (n = 7). Dashed line indicates the contribution from the G_{MAX} increase and from the shift of G(V) respectively. C. Concentration-response for RTG on ΔV_{50} for hKv7.2/7.3 and hKv7.4 (both expressed in *Xenopus* oocytes) at pH 7.4. Data = mean ± SEM. Equation 3 for hKv7.2/7.3: ΔV_{MAX} = −42.1 ± 2.4 mV, EC_{50} = 12.2 ± 2.2 µM (n = 3–5). Equation 3 for hKv7.4: ΔV_{MAX} = −32.9 ± 5.7 mV, EC_{50} = 62.6 ± 22.1 µM (n = 6–7). D. Concentration-response for RTG on ratio of G_{MAX} for hKv7.2/7.3 and hKv7.4 (both expressed in *Xenopus* oocytes) as indicated at pH 7.4. Data = mean ± SEM. Equation 3 to hKv7.4: G_{MAX} = 8.0 ± 0.8, EC_{50} = 17.0 ± 5.6 µM (n = 6–7). E. 30 µM Wu200 on hKv7.4 (B for explanation). The arrow indicated a 2.8-fold increase. F. G(V) curves for recordings as in E. Data = mean ± SEM. Equation 2: V_{50} = −17.3 ± 1.5 mV, −42.3 ± 3.3 mV, ΔV_{50} = −25.0 ± 2.9 mV; G_{MAX} increased by a factor of 1.6 ± 0.1 (n = 7). Dashed lines separate the two effects. G. Wu200 on hKv7.2/7.3 (D for explanation). ΔV_{MAX} = −37.2 ± 1.2 mV, EC_{50} = 11.7 ± 1.4 µM (n = 3–7). Wu200 on hKv7.4: ΔV_{MAX} = −38.1 ± 2.6 mV, EC_{50} = 16.2 ± 3.4 µM (n = 4). H. Wu200 on hKv7.2/7.3 and hKv7.4 (E for explanation). I. G(V) shifts (ΔV_{50}) for hKv7.7/2.7/3 and hKv7.4 (both expressed in *Xenopus* oocytes) at pH 7.4 as indicated. Data = mean ± SEM. Wu161 on hKv7.2/7.3: Data from Figure 1F. Wu161 on hKv7.4: −19.2 ± 2.7 mV (n = 4). Wu199 on hKv7.2/7.3: Data from Figure 3E. Wu199 on hKv7.4: −26.3 ± 1.6 mV (n = 3). Wu200 on hKv7.2/7.3: Data from Figure 3E. Wu200 on hKv7.4: Data from H. RTG on hKv7.2/7.3: Data from Figure 2B. RTG on hKv7.4: Data from C. One-way ANOVA was used with Sidak’s multiple comparison test. ns, p > .05; ****, p < .0001. J. Ratio of G_{MAX} for hKv7.2/7.3 and hKv7.4 (both expressed in *Xenopus* oocytes) at pH 7.4 as indicated. Data = mean ± SEM, n = number of animals. Equation 3: RTG: EC_{50} = 1.74 ± 0.17 µM (n = 6); Wu200: EC_{50} = 14.6 ± 4.4 µM (n = 7). In presence of linopirdine (Lino., 10 µM): RTG+lino: EC_{50} = 20.0 ± 2.6 µM (n = 6); Wu200+lino: EC_{50} = 139.8 ± 56.1 µM (n = 7). L. Concentration dependence for relaxation of mesenteric arteries by indicated compounds. Data = mean ± SEM, n = number of animals. lg EC_{50} (M): Wu161: −5.51 ± 0.16, Wu161 + lino: −4.63 ± 0.16 (n = 7); Wu199: −5.31 ± 0.15, Wu199 + lino: −4.88 ± 0.11 (n = 7); Wu200: −4.80 ± 0.27, Wu200 + lino: −3.67 ± 0.44 (n = 7); RTG: −5.757 ± 0.096, RTG + lino: −4.71 ± 0.12 (n = 6); Paired t-test was used. *, p < .05; **, p < .01; ***, p < .001.
group on a stalk of two atoms (with N in the stalk) did not further increase the induced shifts (Wu24 vs Wu199, and Wu14 vs Wu200), but the compounds still induced a larger shift than the corresponding unhalogenated sulfonyl group compound (Wu109) (Figure 3E). The monohalogenation reduced the EC50 from 51.1 ± 12.6 µM (Wu109) to 12.3 ± 2.2 µM and 11.7 ± 1.3 µM for Wu199 and Wu200, respectively (Figure 3F). (5) Two of the sulfonyl group compounds (Wu181 and Wu200) shifted G(V) as much as RTG at the same concentration (30 µM; Figure 3G).

To summarize, different combinations of a halogenated lipophilic part and a sulfonyl group on a stalk of two atoms (Wu181 and Wu200), can at physiological pH make compounds as potent hKv7.2/7.3 channel activators as RTG. Because the oocytes well tolerated application of high concentrations of Wu161, Wu199, and Wu200, in contrast to Wu181, which made the oocyte membrane leaky,23 they were, therefore, selected for more detailed studies.

2.4 Selectivity between hKv7.2/7.3 and hKv7.4

Due to the low selectivity of RTG among hKv7.2–7.5, RTG also opens hKv7.4 expressed in smooth muscle cells.28 To explore the selectivity for our compounds, we tested RTG and four different resin acid derivatives on hKv7.4. As expected, RTG clearly increased the current through hKv7.4 channels (Figure 4A). However, in contrast to the effects of Wu161 on hKv7.2/7.3 (Figure 1F), where the G(V) shift was the dominating channel-opening effect, the increase in GMAX was here the dominating channel-opening effect (Figure 4B). Although the affinity of RTG on the G(V) shift is, as earlier reported,29 lower for hKv7.4 than for hKv7.2/7.3 (Figure 4C), the effect on GMAX is exceedingly large on hKv7.4 (Figure 4B,D). At 30 µM, the GMAX increased by a factor of 6.1 ± 0.2 (n = 7) (Figure 4B,D). In combination with the G(V) shift, the conductance increase at −20 mV (a physiologically relevant potential for vascular smooth muscle) is a factor of 8.2 ± 0.9 (n = 7) (Figure 4B).

As a comparison, Wu200 activated the hKv7.4 channel (Figure 4E,F) and shifted the G(V) to the same extent as for hKv7.2/7.3 (Figure 4F,G) but with almost no effect on GMAX (Figure 4H). Consequently, the increase in current at −20 mV was much less than for RTG (2.9 ± 0.5 [n = 4] vs 8.2 ± 0.9) (Figure 4B,F). The other investigated resin acid derivatives (Wu161 and Wu199) all followed the same pattern as Wu200: no difference in G(V) shift compared with hKv7.2/7.3 (Figure 4I) and almost no effect on GMAX (Figure 4J).

To investigate any physiological effects of the resin acid derivatives on hKv7 channels in vascular smooth muscle, we examined the ability of the compounds to induce relaxation of rat mesenteric artery. Like RTG, Wu161, Wu199, and Wu200 relaxed preconstricted arteries in a concentration-dependent manner (Figure 4K,L). Although Wu161 and Wu199 had EC50 values similar to those of RTG, Wu200 required higher concentrations to elicit vasorelaxation, suggesting that Wu200 can cause less unwanted smooth muscle relaxation than RTG. The Kv7 channel blocker, linopirdine (10 µM), attenuated the relaxation to all compounds, including RTG (Figure 4K,L), suggesting that at least part of the smooth muscle–relaxing effect by the compounds is caused by activation of Kv7 channels.

2.5 Selectivity among other ion channels

Some of the resin acid derivatives have shown a promising therapeutic profile by activating the hKv7.2/7.3 channel to the same degree as RTG, but with a prominent difference compared to RTG on hKv7.4. We here explored the effects of some of the most potent compounds on potential ion channel liability targets (all known to play important roles in the rhythm generation of the heart): hKv11.1 (=hERG), hNaV1.5, and hCaV1.2. These experiments were carried out on cell lines with high-throughput patch-clamp technology. None of the four explored compounds (Wu161, Wu199, Wu200, and RTG) had any effect on hNaV1.5 channel activation (Figure 5A,B,D,E) in the concentration range explored (0.3–30 µM). Wu161 (at 10–30 µM), Wu199 (at 10–30 µM), and Wu200 (at 30 µM) caused small negative shifts of the hNaV1.5 steady-state inactivation curve (Figure 5C,D,F); if anything, this suggests a reduced excitability at the highest concentrations explored. None of the explored resin acid derivatives had any effect on the hKv11.1 channel activation curve, but RTG caused a small, but significant, negative shift at 3–30 µM (Figure 5G–H). At 30 µM, Wu199, Wu200, and RTG reduced the maximum current of hKv11.1 by 11 ± 4%, 22 ± 6%, and 14 ± 4%, respectively, whereas Wu161 had no effect (Figure 5I). For hCaV1.2, up to 10 µM, none of the four tested compounds had any effect on hCaV1.2 activation (Figure 5J,K). At 30 µM, Wu199 caused a minor positive shift (Figure 5K). For the hCaV1.2 steady-state inactivation, 10 µM Wu200 caused a shift of −4.6 ± 1.0 mV (but no shift at 30 µM), and 30 µM RTG caused a shift of −4.4 ± 0.5 mV (Figure 5L).

2.6 Antiseizure effects in a zebrafish larvae model

Due to their effect as activators of the hKv7.2/7.3 channel, we investigated whether Wu161, Wu199, and Wu200 had antiseizure properties by exploring their effect on a simple but robust antiseizure model: the zebrafish-larvae model.
Under control conditions, almost no electrical activity was registered, but upon application of the chemoconvulsant pentylenetetrazol (PTZ, 15 mM), the electrical activity increased with epileptiform bursts (Figure 6A, for criteria see Section 4). After PTZ treatment there was on average one burst per minute and each burst continued for about 2 s (Figure 6B). To validate the model, three functionally distinct antiseizure drugs were tested: carbamazepine (CBZ), valproic acid (VPA), and retigabine (RTG) (Figure 6C). CBZ did not reduce seizure frequency (Figure 6D), whereas VPA and RTG reduced seizure frequency (Figure 6D), consistent with previous studies. Wu199 and Wu200 were tested at 10 and 30 µM, and Wu161 was tested at 10 µM. Wu161 at 30 µM was not lethal but appeared to have a toxic effect (stunted body growth) in <15% of the larvae and was, therefore, not evaluated at this concentration. Intriguingly,
all compounds had a seizure-reducing effect, as demonstrated by the representative recording in a larva treated with Wu200 (Figure 6A) and by a summary of data for all compounds (Figure 6E). Together, these data demonstrate an antiseizure effect of the compounds, with no obvious difference in potency.
3 | DISCUSSION

In this study we have presented data suggesting that resin acid derivatives interact with the top of the voltage sensor S4, to electrostatically activate the hKv7.2/7.3 channel. The site of action is distinct from sites of two other hKv7.2/7.3 channel activators, RTG and ICA-73. A comparison with previous studies on the Shaker Kv channel suggests that some general principles are shared between different channels while some details differ: (1) The resin acid derivatives electrostatically act on the extracellular end of the voltage sensor S4 in both channels.\(^{21}\) This electrostatic mechanism is also shared with that of polyunsaturated fatty acids on the Shaker Kv, hKv7.1, and hKv7.2 channels.\(^{24,33,34}\) (2) A charge attached to a stalk of two atoms is more efficient than a charge attached directly to an unhalogenated three-ringed lipophilic resin skeleton in both channels.\(^{21}\) (3) Mono-halogenation of C12 or C14 enhanced the activation of hKv7.2/7.3, whereas multiple halogenations had no extra effect or even decreased the effect. This is in contrast to what we found for the 3R Shaker Kv channel.\(^{25}\) (4) A side chain on C7 decreased the enhancing effect by a halogen in hKv7.2/7.3. This is in contrast to the effect on the 3R Shaker Kv channel where a side chain of C7 increased the effect.\(^{25}\) Altogether, this suggests that, despite structural similarities between the VSDs of the hKv7.2/7.3 and the Shaker Kv channel, there are differences in their interactions with resin acid derivatives, indicating possibilities of hKv channel selectivity.

Previous studies on RTG validated hKv7.2/7.3 as an antiseizure target, supporting the development of new hKv7.2/7.3 channel activators. However, the problem with RTG was that it had several side effects, which included urinary retention and postural hypotension.\(^{35}\) Many of these side effects could be attributed to activation of Kv7.4 channels as well as Kv7.5 channels found in smooth muscle cells. Thus when constructing new antiseizure drugs activating hKv7.2/7.3 it is imperative that these drugs avoid activating hKv7.4. Although resin acid derivatives and RTG shift the G(V) curves for both channels in a negative direction along the voltage axis, there are huge differences in the effect on the maximum conductance G\(_\text{MAX}\); RTG has a substantial effect on G\(_\text{MAX}\) of hKv7.4, whereas the resin acid derivatives have almost none. In addition to the large size of RTG’s G\(_\text{MAX}\) increase, there are two other reasons why an effect on the G\(_\text{MAX}\), rather than the G(V) shift of hKv7.4, is critical to explain side effects: (1) The slope of G(V) is shallower for hKv7.4 \((s = 13.1 \pm 0.3 \text{ mV}, n = 69, \text{ see Equation } 2)\) than for hKv7.2/7.3 \((s = 8.5 \pm 0.1 \text{ mV}, n = 152)\), implying that a G(V) shift has a smaller effect on the current amplitude. (2) The resting potential in smooth muscle is more depolarized than in neurons,\(^{36}\) implying that a G(V) shift plays a smaller role. This means that the resin acid derivatives activate the hKv7.4 channel less than RTG at resting potential. Consistent with this, data presented in this investigation show that the resin acid derivatives are less able to relax mesenteric artery segments than RTG.

Similar to the fundamental role of Kv7.2/7.3 channels in setting the membrane potential and to regulate excitability of neurons, Kv7.4 and Kv7.5 channels have a fundamental role in setting the resting potential of vascular and nonvascular smooth muscle cells.\(^{35,37}\) In the vasculature, smooth muscle cells control vascular tone. Activation of the Kv7.4/7.5 channels hyperpolarizes the resting potential, thereby reducing the open probability of voltage-dependent calcium channels and limiting calcium influx. Thus RTG, and several other Kv7 channel activators, cause a vasorelaxation in a number of precontracted murine blood vessels including thoracic aorta, cerebral arteries, and mesenteric arteries, as well as human arteries.\(^{32,35,38}\) Consequently, testing novel compounds on mesenteric artery relaxations is a valuable tool to determine the potential effects on Kv7.4/7.5 channels.\(^{39}\) In addition to the vasculature, Kv7.1, Kv7.4, and Kv7.5 channels have fundamental roles in the smooth muscle of the airways, the uterus, the digestive system (stomach and colon), and bladder.\(^{35}\) As a consequence, activation of Kv7 channels in smooth muscle can, in addition to urinary retention and postural hypotension, also cause constipation, which was another identified side effect by RTG in clinical trials.\(^{40}\) This extensive role for Kv7 channels in smooth muscle highlights the need for more extensive evaluation of the resin acid derivatives on overexpressed Kv7.1 and Kv7.5 channels, and also physiological tests on, for example, bladder contractility.

Finally, we showed that Wu161, Wu199, and Wu200 had robust antiseizure effects in the zebrafish larvae epilepsy model. Both adult and larval zebrafish express functional Kv7 channels in the nervous system and, in their transmembrane parts, the zebrafish Kv7 channels are very similar to human Kv7 channels.\(^{31,41,42}\) The clear antiseizure effects might be surprising because the compounds are permanently charged, with potential problems in penetrating the tissue. One explanation is that the blood-brain barrier in the zebrafish larvae is immature.\(^{41}\) Due to their permanent negative charge, we do not expect these specific resin acid derivatives to traverse membranes, and thus not to be taken as an oral drug or to be able to pass the blood-brain barrier. Consequently, further molecular optimization is needed. However, these resin acid derivatives have antiseizure properties and activate the hKv7.2/7.3 channel by a lipoelectric mechanism, a mechanism of action different from any other antiseizure compounds. A direct electrostatic effect on the movement of the voltage sensor, the channel’s own control system, enables a fine tuning of the voltage dependence; if future molecular modifications increase the affinity of the compound to the channel and the therapeutic effect occurs at the top plateau of the concentration-response curve, then a modification of the distance between the charge of the compound and the voltage sensor S4 can fine-tune the electrostatic effect to...
get a desired therapeutic effect. To our knowledge, these are the first compounds acting by the lipoelectric mechanism that have antiseizure properties.

4 | METHODS

The complete Methods are described in Supplementary Methods.

All animal experiments were approved by Linköping’s (allowance number ID 1941) and Copenhagen’s (allowance number P19-006) local Animal Care and Use Committees. The principles outlined in the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and the Basel declaration https://www.basel-declaration.org/ including the 3R (Replace, Reduce, and Refine) concept have been considered when planning the experiments.

4.1 | Chemical compounds and syntheses

The syntheses of Wu199 and Wu200 are described in Supplementary Methods. The syntheses of compounds Wu13,33 Wu14, 33 Wu24, 33 Wu27,33 Wu32, 33 Wu36, 33 Wu50,31 Wu60, 33 Wu109, 34 Wu161, 34 and Wu181 34 were described in our previous studies.

4.2 | Ion channels, molecular biology, and cell lines

We investigated human (h) voltage-gated Kv7.2, Kv7.3, Kv7.4, Kv11.1 (=hERG), NaV1.5, and CaV1.2 channels. hKv7.2, hKv7.3, and hKv7.4 were expressed and studied in Xenopus laevis oocytes. Site-directed mutagenesis, sequencing, surgery of Xenopus laevis frogs, isolation of oocytes, and storage and injection of oocytes have been described in detail previously.25,44 hKv7.2/7.3 was injected in a 1:1 ratio as described previously.45 The stably expressing cell lines HEK-Kv7.2/7.3 (Saniona), CHO-Kv11.1 Duo (B’SYS), HEK-NaV1.5 (CreaCell), and CHO-CaV1.2 (Charles River Laboratories) were used to study the cells’ currents on the automated patch-clamp system QPatch 48 or Qube 384 (Sophion Bioscience A/S).

4.3 | Two-electrode voltage-clamp recordings

K+ currents were measured with the two-electrode voltage clamp technique (GeneClamp 500B amplifier, Axon instruments; Digidata 1440A converter, Molecular Devices; Clampex 10.5, Molecular Devices) as described previously.25 All experiments were performed at room temperature.

4.4 | Automated patch-clamp technique

Automated patch-clamp recordings were done at room temperature on a QPatch 48 (for hKv7.2/7.3, hKv11.1, and hNaV1.5 currents) or Qube 384 (for hCaV1.2 currents) platform (Sophion Bioscience A/S).

4.5 | Analysis of ion current data

The TEVC data were analyzed using Clampfit 10.5 (Molecular Devices) and GraphPad Prism 8 (GraphPad software). APC data were analyzed using Sophion Analyzer 6.4.

For voltage dependence of activation of hNaV1.5 and hCaV1.2, the conductance, $G(V)$, was calculated as from:

$$G(V) = I / (V - V_{REV})$$

where $V_{REV}$ is the reversal potential and was calculated for each channel.

The $G(V)$ data were fitted to the Boltzmann curve:

$$G(V) = G_{MAX} / (1 + \exp ((V_{50} - V) / s))$$

where $G_{MAX}$ is the estimated maximum conductance of the $G(V)$ curve, $V_{50}$ is the midpoint, and $s$ is the slope value. Concentration dependence for the $G(V)$ (or $I(V)$) shifts (or $G_{MAX}$ or contraction [see below]) were quantified as

$$\Delta V = \Delta V_{MAX} / (1 + E_{C50} / c)^h$$

where $\Delta V$ is the voltage shift, $\Delta V_{MAX}$ is the maximum $\Delta V$, $E_{C50}$ is the half maximal effective concentration, $c$ is the concentration, and $h$ is the Hill slope (set to 1 in all figures but Figure 4K).

4.6 | Isometric tension recordings

Male Wister rats ages 12 to 14 weeks were used in accordance with Directive 2010/63EU on the protection of animals used for scientific purposes and approved by the national ethics committee, Denmark. Rats were group-housed with regular 12-hour light/dark cycles, in clear plastic containers, with ad libitum access to food and water and underwent at least 1 week of habituation. In accordance with the methods of killing animals described in annex IV of the EU Directive 2010/63EU on the protection of animals used for scientific purposes, rats were made unconscious by a single, percussive blow to the head. Immediately after the onset of unconsciousness, cervical dislocation was performed to complete the killing. After euthanasia, the third-order mesenteric arteries were dissected from the rat and placed in ice-cold physiological salt solution.
(PSS). The chambers of the myograph contained PSS maintained at 37°C and aerated with 95% O2/5% CO2. Changes in tension were recorded continuously by PowerLab and Chart software (ADInstruments). The arteries were equilibrated for 30 minutes and normalized to passive force. 46

4.7 | Zebrafish larvae model

Larvae (AB or AB Tg(fli1a:EGFP)y1 strain) (Bioreperia AB) were reared in embryo medium at 28.5°C or at room temperature and used at 3–5 days post fertilization (dpf). No ethical approval was needed for this study, as zebrafish larvae no older than 5 dpf were used. 47 Reference antiseizure drugs were applied at the maximum tolerated dose, the highest concentration at which the zebrafish responded by swimming upon touch provocation after an overnight incubation (18–24 h). 32 The larvae were placed in a recording chamber filled with respective treatment, which was washed out and replaced with 15 mM pentylenetetrazol (PTZ). 30, 48 The local field potential was measured using a glass electrode filled with artificial cerebrospinal fluid inserted into the telencephalon. Epileptiform events and their cumulative duration were analyzed for the last 10 minutes of each recording, representing a state of stabilized seizures. 30 Data analysis was performed in Matlab R2018.b with an automated seizure-detecting algorithm generously provided by Hunyadi and colleagues, 49 and in Clampfit 10.7 (Molecular Devices, LLC). Events were included if the following three criteria were met: (1) complex polyspiking pattern, (2) amplitude ≥3x baseline, and (3) duration ≥1000 msec. 30, 49, 50

4.8 | Statistical analysis

Mean values are expressed as mean ± standard error of the mean (SEM). To compare two different conditions a Student’s t test was used (paired or unpaired dependent of the experimental setup). p < .05 was considered statistically significant for all statistical tests. (* indicates p < .05; ** indicates p < .01; *** indicates p < .001; **** indicates p < .0001.) To compare more than two different conditions, an ordinary one-way or a two-way analysis of variance (ANOVA) (Gaussian distribution of residuals and equal SDs assumed) with appropriate multiple comparison (Tukey’s when comparing all conditions; Dunnett’s when comparing towards a control; Sidak’s when comparing pre-selected pairs). All statistical details for the ANOVAs are presented in Table S1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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