Polymodal thermo- and mechanosensitive two-pore domain potassium (K\textsubscript{2P}) channels of the TREK\textsuperscript{1} subfamily generate 'leak' currents that regulate neuronal excitability, respond to lipids, temperature and mechanical stretch, and influence pain, temperature perception and anaesthetic responses\textsuperscript{1–3}. These dimeric voltage-gated ion channel (VGIC) superfamily members have a unique topology comprising two pore-forming regions per subunit\textsuperscript{4–6}. In contrast to other potassium channels, K\textsubscript{2P} channels use a selectivity filter 'C-type' gate\textsuperscript{7–10} as the principal gating site. Despite recent advances\textsuperscript{11–12}, poor pharmacological profiles of K\textsubscript{2P} channels limit mechanistic and biological studies. Here we describe a class of small-molecule K\textsubscript{2P} activators that directly stimulate the C-type gate by acting as molecular wedges that restrict interdomain movement behind the selectivity filter. Structures of K\textsubscript{2P}2.1 (also known as TREK-1) alone and with two selective K\textsubscript{2P}2.1 (TREK-1) and K\textsubscript{2P}10.1 (TREK-2) activators—an N-aryl-sulfonamide, ML335, and a thiophene-carboxamide, ML402—define a cryptic binding pocket unlike other ion channel small-molecule binding sites and, together with functional studies, identify a cation–π interaction that controls selectivity. Together, our data reveal a druggable K\textsubscript{2P} site that stabilizes the C-type gate 'leak mode' and provide direct evidence for K\textsubscript{2P} selectivity filter gating.

K\textsubscript{2P}2.1\textsubscript{crys} (that is, the construct used for crystallization studies, see Methods for full description; Extended Data Fig. 1a, b) crystalized alone and with activators ML335 (N-(2,4-dichlorophenyl) methyl]-4-(methanesulfonylamo) benzamide) and ML402 (N-[2-(4-chloro-2-methylphenoxy)ethyl]thiophene-2-carboxamide) diffracted X-rays to 3.1 Å, 3.0 Å and 2.8 Å, respectively (Extended Data Table 1) enabling structure determination (Extended Data Fig. 1c). K\textsubscript{2P}2.1\textsubscript{crys} has a domain-swapped M1 helix, extracellular CAP domain, and an unimpeded aqueous path between the intracellular side and selectivity filter, similar to prior TREK subfamily structures\textsuperscript{6,13–15} (Fig. 1a). Features absent in prior K\textsubscript{2P} structures include a C-terminal tail (C-tail) that is five helical turns longer than in K\textsubscript{2P}10.1\textsuperscript{16}, Trp295–Val321 (Fig. 1a, Extended Data Fig. 1c), the 111–128 loop connecting the P1 pore helix and CAP bearing the extracellular pH sensor, His126 (refs 16, 17) (Fig. 1a, Extended Data Fig. 1d), and a set of bound lipids (Extended Data Fig. 1e, f).

Structures of the ML335 and ML402 complexes revealed unambiguous density for two activators per channel (Fig. 1b, Extended Data Fig. 2a–e) occupying an L-shaped pocket behind the selectivity filter formed by the P1 pore helix and M4 transmembrane helix intrasubunit interface (Fig. 1c, d). Notably, and in contrast to the activated mutant K\textsubscript{2P}2.1(G137T)\textsuperscript{7}, K\textsubscript{2P}2.1\textsubscript{crys} responds to both ML335 and ML402 (Extended Data Fig. 2f–i). The ML335/ML402 binding pocket differs from the TREK antagonist norfluroxetine\textsuperscript{8} binding site (Fig. 1e) and is dissimilar to other VGIC superfamily pore domain antagonist sites\textsuperscript{8,19} (Extended Data Fig. 3). Thus, the ML335/ML402 site, dubbed the 'K\textsubscript{2P} modulator pocket', establishes a novel point for VGIC superfamily small molecule modulation.

The K\textsubscript{2P} modulator pocket comprises a 'P1 face' and an 'M4 face' that form a common set of hydrogen bonds, π–π, and cation–π\textsuperscript{10} interactions with ML335 and ML402 (Fig. 2a, b and Extended Data Fig. 4a, b). Both compounds adopt an L-shaped conformation enabling their 'upper ring' (ML335 N-aryl sulfonamide and ML402 thiophene) and 'lower ring' (ML335 dichloro-benzyl and ML402 aryl ether) to engage the P1 helix residue Phe134 through face–face and edge–face interactions, respectively (Fig. 2a, b). On the M4 face, the upper and lower rings make a cation–π interaction with Lys271 and edge–face interaction with Trp275, respectively (Fig. 2, Extended Data Figs 2a, b and 4a, b). The amide groups of both compounds also make hydrogen bonds with the Ala259 carbonyl on the loop connecting the second selectivity filter. The ML335 sulfonamide forms additional interactions with the Gly260 carbonyl on the same loop, the P1 face Ser131 hydroxyl, the 111–128 loop His126 imidazole nitrogen, and Asn147 on the first selectivity filter (Fig. 2a, Extended Data Fig. 4a).

The K\textsubscript{2P} modulator pocket constitutes a cryptic binding site requiring conformational changes centred around Phe134, Lys271 and Trp275 (Fig. 2c–d, Supplementary Video 1). Without activators, Phe134 and Lys271 and Trp275 are mobile in the unliganded structure (Extended Data Figs 5a and 6b), change conformation to form modulator interactions, and together with Phe134 have reduced bound state mobility (Extended Data Fig. 5a–c, and Extended Data Table 2a). Hence, both compounds act as wedges driven into the selectivity filter supporting structure, a mode reminiscent of other channel modulators\textsuperscript{21–22}. Notably, the K\textsubscript{2P} modulator pocket includes gain-of-function mutation sites, Gly137 and Trp275, affecting all TREK subfamily members\textsuperscript{2,8,15,23} (Extended Data Fig. 6b). Together, these observations indicate that the P1–M4 interface is a hub for conformational changes causing TREK subfamily activation and that P1–M4 interface stabilization is central to ML335 and ML402 action.

K\textsubscript{2P} activator pocket residues that contact the compounds are identical among TREK subfamily members, except for the Lys271 cation–π interaction, and diverge in other K\textsubscript{2P} subtypes (Extended Data Fig. 4c, d). Comparison with K\textsubscript{2P}10.1\textsuperscript{16} and K\textsubscript{2P}4.1 (TTRA)K\textsuperscript{4–15} (Extended Data Fig. 6a, Extended Data Table 2b) reveals no selectivity filter conformation differences. This structural similarity supports the idea that ML335 and ML402 influence selectivity filter dynamics, similar to inferences from structural studies of P1–M4 interface gain-of-function mutants\textsuperscript{15}. There are notable differences in the first two M4 helical turns of the K\textsubscript{2P} modulator pocket and the conformations of the Phe134 and Trp275 equivalent (Extended Data Fig. 6b–f).
Given the varied conformations of these residues in the absence of activators and mobility changes between the unliganded and liganded structures (Extended Data Fig. 5), the main action of the activators appears to be to limit the conformations sampled by P1–M4 interface elements.

The K<sub>2P</sub>2.1 C-tail senses phospholipid<sup>24,25</sup>, phosphorylation<sup>26,27</sup>, temperature<sup>28,29</sup>, and pressure<sup>30</sup> gating commands and forms a continuous helix with M4 (Fig. 3a). Prior TREK subfamily structures uncovered varied M4 conformations spanning extremes termed ‘up’ and ‘down’<sup>14,13–15</sup>. Proposals that the up<sup>14</sup>, down<sup>15</sup>, or both conformations are active<sup>6,23</sup>, have been advanced. In all K<sub>2P</sub>2.1 structures, M4 is up (Fig. 1a) and the selectivity filter sites S1–S4 are occupied by potassium ions (Extended Data Fig. 1g). Because both C-tails make lattice contacts (Extended Data Fig. 1g) that can influence M4<sup>13,15</sup>, the K<sub>2P</sub>2.1 structures presented here cannot directly address the controversy regarding M4 status. Nevertheless, the activator-bound selectivity filter is compatible with the up M4 conformation.

The C-tail has two faces, an electropositive patch comprising four residues implicated in PIP<sub>2</sub> modulation (Arg297, Lys301, Lys302 and Lys304)<sup>34,25</sup>, and a face housing the intracellular proton sensor site, Glu306<sup>29</sup>, and inhibitory phosphorylation site, Ser300<sup>29</sup> (Fig. 3a, b). The channel electrostatic profile shows a second positively charged region at the M1–M2–M4 junction (Fig. 3b) suggesting that the resultant interhelical groove may be a phospholipid binding site.

All three K<sub>2P</sub>2.1 structures revealed tubular densities at locations different from previous K<sub>2P</sub> lipid binding sites<sup>4,6,14,15</sup>, denoted as lipids L1, L2, and L3 (Fig. 3c, Extended Data Fig. 1e, f). L1 resides at the M2–P2 inter-subunit junction (Fig. 3c). L2 and L3 are part of a single phospholipid (Extended Data Fig. 1e, f) and sit in the groove formed by the positively charged M1–M2–M4 inter-subunit junction (Fig. 3c, b and Extended Data Fig. 1e, f). The L2–L3 site seems to be a prime point for modulatory lipids, as the positively charged residues that affect PIP<sub>2</sub> responses<sup>24,25</sup> are on the helical face opposite to L2–L3, and M4 conformational changes could affect how these residues interact with regulatory lipid head-groups.

Xenopus oocyte two-electrode voltage-clamp measurements show that ML335 and ML402 activate K<sub>2P</sub>2.1 and K<sub>2P</sub>10.1 but not K<sub>2P</sub>4.1 (14.3 ± 2.7 μM, K<sub>2P</sub>2.1–ML335; 13.7 ± 7.0 μM, K<sub>2P</sub>2.1–ML402; 5.2 ± 0.5 μM, K<sub>2P</sub>10.1–ML335; and 5.9 ± 1.6 μM, K<sub>2P</sub>10.1–ML402) (Fig. 4a–f and Extended Data Fig. 7a–c). The K<sub>2P</sub> modulator pocket has a single difference among TREK subfamily members at the cation–π interaction position, K<sub>2P</sub>2.1 Lys271 (Extended Data Fig. 4c), which is also a lysine in K<sub>2P</sub>10.1 but a glutamine in K<sub>2P</sub>4.1. Hence, we...
Figure 3 | K2P.2.1 C-tail and lipid binding sites. a. K2P.2.1 C-tail. Positively charged residues are blue. S300A and E306A, a site having slight distortion from helical geometry, are magenta. b. K2P.2.1 Electrostatic surface potential. Orange box highlights M1–M2–M4 junction. Cytoplasmic view (right) indicates C-tail positively charged patch. c. Lipids L1, L2 and L3 (cyan and white) show as space filling. ML335 (yellow) is indicated. Insets show lipid binding pocket details.

asked whether this residue controlled the selective actions of ML335 and ML402. Swapping the Lys271 equivalent between K2P2.1 and K2P4.1 resulted in a clear phenotype reversal for ML335 and M402. (Extended Data Fig. 7d–g). K2P2.1(K271Q) was insensitive to ML335 and ML402, whereas K2P4.1(Q258K) responded to both with a similar EC50 to K2P2.1(14.3 ± 2.7 μM, K2P2.1–ML35; 16.2 ± 3.0 μM, K2P4.1(Q258K)–ML35; 13.7 ± 0.7 μM, K2P2.1–ML402; 13.6 ± 1.5 μM, K2P4.1(Q258K)–ML402) but with a lower magnitude response than K2P2.1. Notably, the effects of TREK subfamily activators arachidonic acid (27,30), BL-124931,32, and ML67-3316 were unchanged (Extended Data Fig. 7h–k). To probe the cation–π interaction further, we synthesized a ML335 congener, ML335A, in which the aromatic upper ring was replaced with an aliphatic ring (Fig. 4c). ML335A had no effect on K2P2.1 (Fig. 4c), supporting the importance of the cation–π interaction. Together, these data identify the Lys271 cation–π interaction as the origin of ML335/ML402 subtype selectivity and establish that this interaction is essential for activation.

The principal K2P channel gating site is the selectivity filter C-type gate9,10. This gate is highly sensitive to permeant ions8,16,17 and is thought to function via 'flux gating' whereby outward, but not inward, ion flow stabilizes the active conformation8. Physiological K2P2.1 activators, such as arachidonic acid, PIP2, and intracellular acidification, shift the channel from outward rectifier mode to an ohmic 'leak mode'. Because the K2P modulator pocket contains architectural elements that support the activator selectivity and gain-of-function mutations that activate the C-type gate8,11, we asked whether structural changes to the P1–M4 interface caused by ML335, ML402, or gain-of-function mutations affect C-type gate function.

Measurement of K2P2.1 in inside-out patches under conditions that potentiate flux-dependent C-type gate activation (150 mM K+<sub>out</sub> versus 150 mM Rb+<sub>in</sub>)9 showed the expected outward rectification (Fig. 4g, k).
ML335 and ML402 activate \( \text{K}_{\text{Tp}} \)2.1 in HEK293 cells similar to their effects in Xenopus oocytes (5.2 ± 0.8 pM and 5.9 ± 1.6 pM for ML335 and ML402, respectively (n ≥ 3)) (Figs. 4g–i and Extended Data Fig. 8a). Compound application essentially eliminated flux-dependent outward rectification (Fig. 4h, i, k) yielding a rectification coefficient \( (I_{\text{c,000 mV}}/I_{\text{c,000 mV}}) \) of approximately 1. This outcome matches physiological activator effects and establishes that ML335 and ML402 activate the C-type gate. The \( \text{K}_{\text{Tp}} \)4.1 gain-of-function mutation GI241 reshapes the \( \text{K}_{\text{Tp}} \) modulator pocket through structural consequences similar to ML335/ML402, namely an outward M4 movement and repositioning of the \( \text{K}_{\text{Tp}} \)2.1 Phe134 equivalent residue (Extended Data Fig. 6f). \( \text{K}_{\text{Tp}} \)2.1(G1371) and \( \text{K}_{\text{Tp}} \)4.1(G1241) \( \) caused a similar mode shift and rectification coefficient change to ML335 and ML402 (Fig. 4j, k and Extended Data Fig. 8b–d) establishing that \( \text{K}_{\text{Tp}} \) modulator pocket manipulation by ML335/ML402 binding or motion directly activates the selectivity filter C-type gate. Together with the compound-binding-induced mobility reduction of the P1–M4 interface (Extended Data Fig. 5) and lack of \( \text{K}_{\text{Tp}} \) selectivity filter structural differences (Extended Data Fig. 6a), our findings strongly support the idea that direct C-type gate activators stimulate function by reducing the dynamics of the selectivity filter and surrounding structure.

The roles of TREK channels in ischaemia, pain, analgesia and anaesthetic responses suggest that TREK activators could provide new avenues for neuroprotection and pain control. Many natural TREK activators are thought to act on the C-tail and influence the C-type gate through M4\( ^{6,18,19} \), the movement of which is targeted by the antagonist norfluoxetine\( ^{23} \). In this regard, ML335 and ML402 represent a new \( \text{K}_{\text{Tp}} \) modulator class, as they function by binding directly to a pocket at the heart of the channel active site, the C-type gate, rather than influencing the C-tail or M4.

The \( \text{K}_{\text{Tp}} \) modulator pocket defines the first VGIC superfamily pore domain small-molecule activator site and differs from antagonist sites occupying lateral fenestrations below the selectivity filter\( ^{6,18} \) (Fig. 1e and Extended Data Fig. 3a). Voltage-gated calcium channel antagonists, amloidipine and nifedipine\( ^{6,18} \), act at an interface similar to the \( \text{K}_{\text{Tp}} \) modulator pocket, but at a pore domain outer rim site normally occupied by lipids\( ^{6,18} \) (Extended Data Fig. 3b). Notably, all of these small-molecule sites are found at interfaces that are thought to move during gating\( ^{6,18} \), highlighting the potential of channel inter-subunit interfaces as small-molecule control sites.

Our findings provide a structural basis for understanding \( \text{K}_{\text{Tp}} \) C-type gate activation. The \( \text{K}_{\text{Tp}} \) modulator pocket in unliganded \( \text{K}_{\text{Tp}} \)2.1 displays mobility in key elements that is reduced upon activator engagement\( ^{6,18} \) (Fig. 4l, Extended Data Fig. 5 and Extended Data Table 2a) and causes the filter to enter the leak mode (Fig. 4h, i), bypassing modulation mechanisms that may involve other channel regions. The \( \text{K}_{\text{Tp}} \) modulator pocket properties revealed by our studies raise the possibility that natural processes or native signalling molecules may also target this site.

Opening the \( \text{K}_{\text{Tp}} \) modulator pocket requires small movements of few residues, similar to soluble protein cystic modulator sites\( ^{33} \). \( \text{K}_{\text{Tp}} \) modulator pocket diversity (Extended Data Fig. 4c, d), the P1–M4 interface susceptibility to gain-of-function mutations\( ^{7,8} \), and demonstration that a single residue therein can define modulator selectivity suggests that this site may be amenable for \( \text{K}_{\text{Tp}} \) subtype-selective pharmacology development. As the fundamental pocket architecture is conserved in the VGIC superfamily, similar modulatory mechanisms may exist in other superfamily members where selectivity-filter-based gating is central. Thus, this site should provide a fertile target for channel modulator discovery.

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8. The roles of TREK channels in ischaemia, pain, analgesia and anaesthetic responses suggest that TREK activators could provide new avenues for neuroprotection and pain control. Many natural TREK regulators are thought to act on the C-tail and influence the C-type gate through M4\( ^{6,18} \). The movement of which is targeted by the antagonist norfluoxetine\( ^{23} \). In this regard, ML335 and ML402 represent a new \( \text{K}_{\text{Tp}} \) modulator class, as they function by binding directly to a pocket at the heart of the channel active site, the C-type gate, rather than influencing the C-tail or M4.

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Our findings provide a structural basis for understanding \( \text{K}_{\text{Tp}} \) C-type gate activation. The \( \text{K}_{\text{Tp}} \) modulator pocket in unliganded \( \text{K}_{\text{Tp}} \)2.1 displays mobility in key elements that is reduced upon activator engagement\( ^{6,18} \). The \( \text{K}_{\text{Tp}} \) modulator pocket complexes, but ‘down’ for \( \text{K}_{\text{Tp}} \)4.1(G1241), the data support the idea that M4 position is not the sole determinant of channel state\( ^{23} \). The importance of changes in P1–M4 interface dynamics explain how M4 can affect channel function and allow the up or down conformations to activate the channel\( ^{23} \), as both states could limit the mobility of the P1/M4 interface. Such plasticity may be important for enabling TREK subfamily polymodal modulation. Our findings indicate that under basal conditions, \( \text{K}_{\text{Tp}} \)2.1 equilibrates between a resting state having a mobile P1–M4 interface and an activated state in which the mobility of this site is limited. ML335 and ML402 directly stabilize the C-type gate by acting like molecular wedges that reduce P1–M4 interface dynamics (Fig. 4l, Extended Data Fig. 5 and Extended Data Table 2a) and cause the filter to enter the leak mode (Fig. 4h, i), bypassing modulation mechanisms that may involve other channel regions. The \( \text{K}_{\text{Tp}} \) modulator pocket properties revealed by our studies raise the possibility that natural processes or native signalling molecules may also target this site.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** M.L., C.A., C.B. and D.L.M. conceived the study and designed the experiments. T.M. and Y.S. conceived and ran thallium flux assays. M.L. and C.A. performed experiments. M.L. and K.A.C. expressed and purified proteins. M.L. performed crystallization and structure determination. M.L. and C.A. performed electrophysiological experiments and analysed the data. C.B. designed synthetic routes, synthesized, and purified the compounds. D.L.M. analysed data and provided guidance and support. M.L., C.A. and D.L.M. wrote the paper.

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METHODS

No statistical methods were used to predetermine sample size. No randomization or blinding was used.

Constrict screening. A set of mutants and deletion constructs of mouse K2P2.1 bearing a C-terminal green fluorescent protein (GFP) and His6 tag was expressed from a previously described Pichia pastoris pPICZ vector. Plasmids were linearized with Pmel and transformed into P. pastoris SMD1163H by electroporation. Multi-integration recombinants were selected by pPICZ vector. Plasmids were linearized with HindIII and used for transformation into competent E. coli DH5α (str R, thi, supE44). Plasmid pPICZαA was digested with HindIII and treated with T4 DNA polymerase to make a blunt ended fragment. This DNA fragment was then ligated to the linearized plasmid pPICZαA. The EcoRI fragment containing the gene was amplified by PCR using plasmid pPICZαA as a template and primers 5′-GGGAATTCATATGAGCTCGAGCTTTGAGTGGTACCC-3′ and 5′-GGGCAATTCTACGAGTAGGGGCTTGTGGCCATC-3′, which contain the EcoRI and HindIII sites, respectively. The PCR product was purified by gel extraction, digested with EcoRI and HindIII, and cloned into the expression vector pPICZαA to yield pPICZαA (His6)-K2P2.1cryst. The purified recombinant K2P2.1cryst was expressed in Pichia pastoris SMD1163H. The protein was recovered from the periplasmic fraction of the recombinant strain by lysis and centrifugation at 15,000 g. The supernatant was subsequently dialyzed against 200 mM KCl, 2.1 mM OGNG, 15 mM HTG, 0.012% CHS, 20 mM MES, pH 6.0, for 24–48 h at 4 °C.

Protein expression. K2P2.1cryst bearing a C-terminal green fluorescent protein (GFP) and His6 tag was expressed from a previously described Pichia pastoris pPICZ vector. Plasmids were linearized with Pmel and transformed into P. pastoris SMD1163H by electrophoration. Multi-integration recombinants were selected by pPICZ vector. Plasmids were linearized with HindIII and used for transformation into competent E. coli DH5α (str R, thi, supE44). Plasmid pPICZαA was digested with HindIII and treated with T4 DNA polymerase to make a blunt ended fragment. This DNA fragment was then ligated to the linearized plasmid pPICZαA. The EcoRI fragment containing the gene was amplified by PCR using plasmid pPICZαA as a template and primers 5′-GGGAATTCATATGAGCTCGAGCTTTGAGTGGTACCC-3′ and 5′-GGGCAATTCTACGAGTAGGGGCTTGTGGCCATC-3′, which contain the EcoRI and HindIII sites, respectively. The PCR product was purified by gel extraction, digested with EcoRI and HindIII, and cloned into the expression vector pPICZαA to yield pPICZαA (His6)-K2P2.1cryst. The purified recombinant K2P2.1cryst was expressed in Pichia pastoris SMD1163H. The protein was recovered from the periplasmic fraction of the recombinant strain by lysis and centrifugation at 15,000 g. The supernatant was subsequently dialyzed against 200 mM KCl, 2.1 mM OGNG, 15 mM HTG, 0.012% CHS, 20 mM MES, pH 6.0, for 24–48 h at 4 °C.

Protein purification. In a typical preparation, 50 g of cells were broken by cryo-milling (Retech model MM400) in liquid nitrogen (5 × 3 min, 25 Hz). All subsequent purification was carried out at 4 °C. Cell powder was added at a ratio of 1 g cell powder to 3 ml lysis buffer (200 mM KCl, 21 mM OGNG, 30 mM HEPES (pH 7.2), 1 mg ml −1 -d-thioglucopyranoside, 250 g l −1 Na₂SO₄, 14.9 g l −1 MgSO₄·7H₂O, 9.9 g l −1 (NH₄)₂SO₄, 25 g l −1 hexamethaphosphate, 4.25 ml l −1 PTM, 0.5 ml l −1 of Matrigel (BD Biosciences). The cell lysate was centrifuged at 10,000 g for 4 h at 4 °C. The supernatant was filtered through a 0.22-μm filter and applied to a Superdex 200 gel filtration column equilibrated in SEC buffer.

Protein sample was concentrated using 100-kDa cut-off membranes and then applied to a Superdex 200 gel filtration column equilibrated in SEC buffer.

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was 0.2%). Other concentrations were prepared by serial dilutions of the 100μM solution in recording buffer supplemented with 0.1% DMSO.

Xenopus oocytes were collected in compliance with ethical regulations specified by the UCSF Institutional Animal Care and Use Committee protocol AN129690.

Synthetic chemistry. Detailed descriptions of synthesis routes and characterization of ML335, ML335a and ML402 are found in the supplementary material.

Data availability. Coordinates and structure factors have been deposited with the Protein Data Bank under accession codes 5VK5 (K$_{2}$P.2.1), 5VKN (K$_{2}$P.2.1–ML335) and 5VKP (K$_{2}$P.2.1–ML402).

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Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | K$_{2P}$.1$_{cryst}$ function and structure. a, Exemplar recording from K$_{2P}$.1$_{cryst}$ expressed in Xenopus oocytes. Current was elicited from a $-80$ mV holding potential followed by a 500 ms ramp from $-150$ mV to $+50$ mV. b, K$_{2P}$.1$_{cryst}$ potassium selectivity recorded in Xenopus oocytes in K$^+/$N-methyl-D-glucamine solutions (98.0 mM total) at pH$_o$ = 7.4. Data represent mean ± s.e.m. ($n$ = 4). Dashed grey line represents Nernst equation $E_{rev} = RT/F \times \log([K^+]_o/[K^+]_i)$, where $R$ and $F$ have their usual thermodynamic meanings, $z$ is equal to 1, and $T = 23$ °C, assuming [K$^+$]$_i$ = 108.6 mM (ref. 48). c, Exemplar 2$F_o - F_c$ electron density (1.0σ) for the C-tail region of K$_{2P}$.1$_{cryst}$. Select residues and channel elements are indicated. d, Extracellular view of K$_{2P}$.1$_{cryst}$ showing environment of His126 and Ile148 (purple). Select residues are labelled. The extracellular proton sensor His126$^{16,17}$ is supported by a highly conserved residue, Trp127, and contacts a gain-of-function mutant site, Ile148$^g$, that interacts with the selectivity filter residue Asn147. This network of physical interactions indicates how changes at His126$^{16,17}$ or Ile148$^g$ could affect the C-type gate. e, f, Exemplar L2/L3 lipid electron density for K$_{2P}$.1–ML335. 2$F_o - F_c$ (e; blue, 1.0σ) and $F_o - F_c$ (f; pink, 3.0σ). Chains are coloured smudge and light orange. Channel elements and select residues are labelled. g, Crystal lattice packing for K$_{2P}$.1$_{cryst}$ showing that the C-tail makes lattice interactions stabilized by a cadmium ion coordinated between His313 of adjacent symmetry mates. Asymmetric unit is coloured smudge (chain A) and orange (chain B). Symmetry related channels are shown in slate (chain A) and cyan (chain B). Insets show the anomalous difference map (5.0σ) and locations of Cd$^{2+}$ ions and their ligands. Colours defined according to PyMol, see https://pymolwiki.org/index.php/Color_Values for reference.
Extended Data Figure 2 | \( K_{\text{ap}2.1} \) modulator binding pocket densities and \( K_{\text{ap}2.1} \) functional properties. a–e. Exemplar electron densities for the modulator binding pockets. a–c, 2\( F_o \) – \( F_c \) densities (blue) for \( K_{\text{ap}2.1} \)–ML335 (a; 1.5\( \sigma \)), \( K_{\text{ap}2.1} \)–ML402 (b; 1.0\( \sigma \)) and \( K_{\text{ap}2.1} \) (c; 1.0\( \sigma \)). Offset angle for the cation–\( \pi \) interactions for Lys271–ML335 and Lys271–ML402 is shown and adopts an oblique geometry common to cation–\( \pi \) interactions. d, e, \( F_o \) – \( F_c \) densities (pink, 3.0\( \sigma \)) for \( K_{\text{ap}2.1} \)–ML335 (d) and \( K_{\text{ap}2.1} \)–ML402 (e). f–h. Exemplar current traces for \( K_{\text{ap}2.1} \) (black) with 40\( \mu \)M ML335 (orange) (f), \( K_{\text{ap}2.1} \) (black) with 80\( \mu \)M ML402 (green) (g) and \( K_{\text{ap}2.1} \) (black) with 80\( \mu \)M ML335 (blue) (h). i. Dose–response curves for \( K_{\text{ap}2.1} \)–ML335 (black), \( EC_{50} = 14.3 \pm 2.7 \mu \)M (n \( \geq \) 5); \( K_{\text{ap}2.1} \)–ML402, \( EC_{50} = 14.9 \pm 1.6 \mu \)M (n \( \geq \) 3); \( K_{\text{ap}2.1} \) (black) with 80\( \mu \)M ML335 (yellow orange) (i). © 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Extended Data Figure 3 | Comparison of K<sub>2P</sub> modulator and VGIC antagonist sites. <b>a</b>, Superposition of the K<sub>2P.1</sub>–ML335 complex (smudge and orange) with the BacNaV ‘pore-only’ NaVMs structure<sup>18</sup> (magenta and pink). Bromine site (Br) from labelled sodium channel antagonists is shown as a firebrick sphere. <b>b</b>, Superposition of the pore domains of the K<sub>2P.1</sub>–ML335 complex (smudge and orange) with the pore domain of the BacNaV CavAb (5KMD) bound to the inhibitor amlodipine (AMLOD)<sup>19</sup>, a site normally occupied by lipid<sup>19,51</sup>. Select residues of the K<sub>2P</sub> modulator pocket are shown as sticks and are labelled. CavAb subunits are coloured cyan, marine, slate and dark blue. ML335 (yellow) and amlodipine (cyan) are shown in space filling representation.
Extended Data Figure 4 | $\text{K}_\text{2P}$ modulator pocket structure and conservation. a, b, Details of ML335 (a) and ML402 (b) interactions with $\text{K}_\text{2P}$.1. c, d, Representative $\text{K}_\text{2P}$ channel sequence comparisons for the M4 face (c) and P1 face (d). Purple bar and orange shading on sequence identifiers denotes the thermo- and mechanosensitive $\text{K}_\text{2P}$.1 subfamily. Protein secondary structure is marked above the sequences. Selectivity filter region is in red. Residues involved in direct interactions with ML335 and ML402 are orange and marked with an orange asterisk. Conserved positions are highlighted. $\text{K}_\text{2P}$.1 is the mouse protein used for this study. $\text{K}_\text{2P}$.1H (TREK-1) is the human homologue. All other $\text{K}_\text{2P}$ sequences are human origin. Sequences and identifiers are as follows: $\text{K}_\text{2P}$.1, NP_034737.2; human $\text{K}_\text{2P}$.2.1, NP_001017424.1; $\text{K}_\text{2P}$.3.1 (TASK-1), NP_002237.1; $\text{K}_\text{2P}$.9.1 (TASK-3), NP_001269463.1; $\text{K}_\text{2P}$.5.1 (TASK-2), NP_071753.2; $\text{K}_\text{2P}$.1.1 (TWIK-1), NP_071337.2; $\text{K}_\text{2P}$.6.1 (TWIK-2), NP_071338.2; $\text{K}_\text{2P}$.7.1 (KCNK7), AA03812.2; $\text{K}_\text{2P}$.16.1 (TALK-1), NP_001128577.1; $\text{K}_\text{2P}$.17.1 (TALK-2), NP_113648.2; $\text{K}_\text{2P}$.12.1 (TALK-3), NP_071337.2; $\text{K}_\text{2P}$.15.1 (TALK-5), NP_071753.2; and $\text{K}_\text{2P}$.18.1 (TRESK), NP_862823.1. The dot in the $\text{K}_\text{2P}$.16.1 (TALK-1) sequence in c denotes the following, non-conserved sequence that was removed to avoid a long alignment gap: NFITPSGLPQEQFQTPHGKPIESQIQIP.
**Extended Data Figure 5 | K<sub>2P</sub> structure comparisons.** K<sub>2P</sub> modulator pocket views coloured by B-factor for K<sub>2P</sub>2.1 (a), K<sub>2P</sub>2.1–ML335 (b) and K<sub>2P</sub>2.1–ML402 (c). Bars show B-factor scale.
Extended Data Figure 6 | K<sub>2P</sub> structure comparisons. a, Backbone atom superposition of K<sub>2P</sub>2.1 (smudge, up), K<sub>2P</sub>2.1–ML335 (yellow, up), K<sub>2P</sub>2.1–ML402 (cyan, up), K<sub>2P</sub>10.1 (4BW5) (pink, up)<sup>6</sup>, K<sub>2P</sub>10.1–norfluoxetine (4XDK) (purple, down)<sup>6</sup>, K<sub>2P</sub>4.1 (4I9W) (limon, up)<sup>13</sup>, K<sub>2P</sub>4.1(G124I) (4RUE) (marine, down)<sup>15</sup>, and K<sub>2P</sub>4.1(W262S) (4RUF) (lime green, down)<sup>15</sup>. ‘Up’ or ‘down’ denotes M4 conformation. Selectivity filter ions for K<sub>2P</sub>2.1 (smudge), K<sub>2P</sub>2.1–ML335 (yellow), and K<sub>2P</sub>2.1–ML402 (cyan) are shown as spheres. ML335 and ML402 are shown as sticks. Select channel elements are labelled.

b, Superposition showing K<sub>2P</sub>2.1 chain A (smudge) and chain B (light orange). Sites of gain-of-function mutations, G137I (orange)<sup>7</sup>, and Trp275<sup>8</sup> are indicated. c, K<sub>2P</sub>2.1–ML335 chain A (pink) and chain B (deep salmon), d, K<sub>2P</sub>2.1–ML402 chain A (blue) and chain B (teal), e, K<sub>2P</sub>2.1 chain A (smudge) and chain B (orange), K<sub>2P</sub>10.1 (4BW5) (pink)<sup>6</sup>, K<sub>2P</sub>10.1 (4XDK) (purple)<sup>6</sup>, f, K<sub>2P</sub>2.1–ML335 (pink), K<sub>2P</sub>4.1 (4I9W) (limon)<sup>13</sup>, K<sub>2P</sub>4.1(G124I) (4RUE) (blue)<sup>15</sup>, K<sub>2P</sub>4.1(W262S) (4RUF) (lime green)<sup>15</sup>. G124I from K<sub>2P</sub>4.1(G124I)<sup>15</sup> is shown as sticks. In b–f, Phe134, His126, Lys271, Trp275 and their equivalents in K<sub>2P</sub>10.1, K<sub>2P</sub>4.1 and K<sub>2P</sub>4.1(G124I), are shown as sticks. In c and d, ML335 and ML402 are shown as sticks.
Extended Data Figure 7 | K_{2P} activator responses. a, b, Exemplar current traces for K_{2P}10.1 (black) with 20μM ML335 (yellow orange) (a) and K_{2P}10.1 (black) with 20μM ML402 (cyan) (b). c, Dose–response curves for K_{2P}10.1 with ML335 (EC_{50} = 5.2 ± 0.5μM (n > 3)) (yellow orange) and ML402 (EC_{50} = 5.9 ± 1.6μM (n ≥ 4) (cyan). d–g, Exemplar current traces for K_{2P}2.1 K271Q (black) and with 20μM ML335 (purple) (d); K_{2P}4.1(Q258K) (black) and with 50μM ML335 (orange) (e); K_{2P}2.1(K271Q) (black) and with 50μM ML402 (purple) (f); K_{2P}4.1(Q258K) (black) and with 50μM ML402 (orange) (g). Currents were evoked from Xenopus oocytes expressing the indicated channels from a −80 mV holding potential followed by a 500 ms ramp from −150 mV to +50 mV. Compound structures are shown. h, i, Exemplar current traces for HEK293 cell inside-out patches expressing K_{2P}2.1 (h) and K_{2P}2.1(K271Q) (i) to stimulation by 10μM arachidonic acid (AA) (green). j, Current potentiation measured in HEK cells at 0 mV in response to 10μM arachidonic acid for K_{2P}2.1 (n = 5) and K_{2P}2.1(K271Q) (n = 4). k, Current potentiation measured in Xenopus oocytes at 0 mV for K_{2P}4.1 (white), K_{2P}2.1 (black), K_{2P}4.1(Q258K) (cyan) and K_{2P}2.1(K271Q) (grey) in response to 10μM BL-1249, 30μM ML67-33, and 20μM ML335. For all experiments (n ≥ 4). Data are mean ± s.e.m.
Extended Data Figure 8 | K²P channel patch clamp recordings. a, Dose response for K²P2.1 to ML335 (black circles) and ML402 (open circles) measured in HEK293 cells by whole-cell patch clamp. EC₅₀ values are 5.2 ± 0.8 μM and 5.9 ± 1.6 μM for ML335 and ML402, respectively (n ≥ 3). b, c, Representative current traces and voltage–current relationship from inside-out patches on HEK293 cells expressing K²P4.1 (b) and K²P4.1(G124I) (c) with a 350-ms voltage-step protocol from −100 mV to +100 mV in 150 mM K⁺[out]/150 mM Rb⁺. d, Rectification coefficients (I_{+100 mV}/I_{−100 mV}) calculated from n ≥ 3 current recordings obtained from the same conditions in b and c.
### Extended Data Table 1 | Data collection and refinement statistics

|                     | K$_{2p}2.1$(TREK-1) (5VK5) | K$_{2p}2.1$(TREK-1): ML355 (5VKN) | K$_{2p}2.1$ (TREK-1): ML402 (5VKP) |
|---------------------|-----------------------------|----------------------------------|----------------------------------|
| **Data collection** |                             |                                  |                                  |
| Space group         | $P2_12_1$                   | $P2_12_1$                        | $P2_12_1$                        |
| Cell dimensions     |                             |                                  |                                  |
| $a$, $b$, $c$ (Å)   | 66.72/120.42 /126.44        | 67.07/119.39/128.18              | 67.09 / 119.56 / 127.21          |
| $\alpha$, $\beta$, $\gamma$ (°) | 90.0 / 90.0 / 90.0 | 90.0 / 90.0 / 90.0 | 90.0 / 90.0 / 90.0 |
| Resolution (Å)      | 87.2 – 3.10 (3.21-3.10)    | 87.4 – 3.0 (3.11 – 3.0)          | 87.1 – 2.8 (2.99 – 2.8)          |
| $R_{merge}$ (%)     | 10.38 (>100%)               | 23.7 (>100%)                    | 14.2 (>100%)                     |
| $I/\sigma(I)$       | 13.35 (0.3)                 | 9.78 (0.66)                     | 11.13 (0.37)                     |
| $CC_{1/2}$          | 0.999 (0.065)               | 0.998 (0.171)                   | 0.999 (0.108)                    |
| Completeness (%)    | 97.0 (100.0)                | 97.0 (100.0)                    | 98.0 (100.0)                     |
| Redundancy (%)      | 6.0 (6.2)                   | 12.9 (13.3)                     | 12.8 (13.6)                      |
| **Refinement**      |                             |                                  |                                  |
| Resolution (Å)      | 15.0 – 3.10 (3.21-3.10)    | 15.0 – 3.0 (3.11 – 3.0)          | 15.0 – 2.8 (2.99 – 2.8)          |
| No. reflections     | 18506                       | 20686                           | 25882                           |
| $R_{work}$ / $R_{free}$ | 26.1 / 31.4                  | 25.8 / 28.3                      | 26.9 / 31.4                      |
| No. atoms           |                             |                                  |                                  |
| Protein             | 4289                        | 4357                            | 4328                            |
| Ligand/ion          | 74                          | 200                             | 174                             |
| K$^+$               | 6                           | 6                               | 6                               |
| Cd$^{2+}$           | 3                           | 3                               | 1                               |
| Lipid               | 65                          | 191                             | 167                             |
| ML355 or ML402      |                             |                                  |                                  |
| Water               | 0                           | 0                               | 0                               |
| **B factors**       |                             |                                  |                                  |
| Protein             | 154.6                       | 107.4                           | 147.7                           |
| Ligand/ion          | 168.7                       | 96.5                            | 131.0                           |
| Water               | n/a                         | n/a                             | n/a                             |
| **R.m.s. deviations** |                             |                                  |                                  |
| Bond lengths (Å)    | 0.002                       | 0.006                           | 0.002                           |
| Bond angles (°)     | 0.480                       | 0.685                           | 0.483                           |

Values in parentheses are for highest-resolution shell. Each dataset was derived from a single crystal.
### Extended Data Table 2 | K2P2.1 B-factor and structure comparisons

| Location               | Residue | K2P2.1 (TREK-1) | K2P2.1 (TREK-1):ML335 | K2P2.1 (TREK-1):ML402 |
|------------------------|---------|-----------------|-----------------------|-----------------------|
| Selectivity filter     | Gly144  | 108.6           | 63.0                  | 92.7                  |
|                        | Phe145  | 109.7           | 64.7                  | 98.4                  |
|                        | Gly146  | 118.5           | 76.5                  | 127.4                 |
| Modulator pocket       | Phe134  | 139.3           | 74.0                  | 104.1                 |
|                        | Lys271  | 181.9           | 83.0                  | 116.1                 |
|                        | Trp275  | 156.5           | 69.9                  | 103.2                 |
| Channel core           |         | 132.4           | 83.5                  | 118.8                 |

### Extended Data Table 2b | Structural comparisons with K2P2.1(TREK-1)

| PDB code | KSP | Co RMSD (Å) (ΔCAP, ΔM4) | Co RMSD (Å) Channel core (ΔCAP, ΔM2, ΔM4) |
|----------|-----|--------------------------|------------------------------------------|
| 5VKN     | K2P2.1(TREK-1):ML335 | 0.443 | 0.388 |
| 5VKG     | K2P2.1(TREK-1):ML402 | 0.462 | 0.426 |
| 4XDK     | K2P10.1(TREK-2):Norfluoxetine | 1.976 | 1.317 |
| 4BW5     | K2P10.1(TREK-2):M4 up | 1.054 | 0.918 |
| 4XDJ     | K2P10.1(TREK-2):M4 down | 1.790 | 1.331 |
| 4IW5     | K2P4.1(TRAAK) | 1.176 | 1.144 |
| 3UM7     | K2P4.1(TRAAK) (no domain swap) | 1.468 | 1.390 |
| 4RUF     | K2P4.1(TRAAK):W262S | 1.351 | 1.288 |
| 4RUE     | K2P4.1(TRAAK):G124I | 1.466 | 1.490 |
| 4WFE     | K2P4.1(TRAAK):M4 down | 1.155 | 1.217 |
| 4WFE     | K2P4.1(TRAAK):M4 up | 1.149 | 1.023 |

a. B-factor comparisons. Selectivity-filter-residue B-factors are below the average channel-core B-factor in both the K2P2.1 and modulator-bound structures. Modulator-pocket-residue B-factors drop relative to the average B-factor in both the ML335 and ML402 complexes, indicating that modulator binding reduces the mobility of these residues. The structures are determined in ~200 mM potassium, a concentration that is expected to stabilize the conformation of the selectivity filter (compare with ref. 47) and that could mask mobility changes. Average B-factor is calculated using the channel core elements on both chains: M1 (residues 47–65), P1 through common part of M2 (residues 127–188), common part of M3 through common part M4 (residues 210–300). The selectivity filter was included in the calculation. Blue and red values are >10% below or above the average B-factor, respectively.

b. KSP channel structure comparisons. Root mean square deviations (RMSDs) are calculated using the following K2P2.1 (5VKN) chain A and chain B elements: (ΔCAP, ΔM4); M1 through cap (residues 47–69), P1 through common part of M4 (residues 127–281); channel core (ΔCAP, ΔM2, ΔM4); M1 (residues 47–65), P1 through common part of M2 (residues 127–188); common part of M3 through common part M4 (residues 210–300). The selectivity filter was included in the calculation. For the non-domain-swapped K2P4.1 (3UM7), residues 47–69 of chain A were compared to equivalent chain B residues.
**Synthetic chemistry**

ML335 and ML402 were identified as K_{2p2.1}(TREK-1) activators using a combination of fluorescence-based thallium flux and automated patch-clamp assays. ML335, ML402, and ML335a were synthesized using chemical synthesis procedures outlined below. ML335 was also purchased from Chembridge.

**Scheme 1: Synthesis of ML335**

![Scheme 1](image)

**Step 1: Preparation of N-[(2,4-dichlorophenyl)methyl]-4-methanesulfonamidobenzamide (ML335)**

A 50 ml reaction bottle equipped with a magnetic stirbar was charged with 2,4-dichlorobenzylamine (100 µl, 0.7 mmol, 1.0 equiv.), 4-(methanesulfonylamino)benzoic acid (0.160 g, 0.7 mmol, 1.0 equiv.) and N,N-dimethylformamide (2.000 ml). To the mixture was added HATU (0.424 g, 1.1 mmol, 1.5 equiv.) followed by triethylamine (0.311 ml, 2.2 mmol, 3.0 equiv.) The reaction mixture was stirred for 3 hours at room temperature and then partitioned between ethyl acetate and 10% aqueous citric acid. The organic layer was separated and washed with brine, then dried over Na₂SO₄, decanted, and concentrated. The crude residue was re-suspended in dichloromethane and purified by automated silica gel flash chromatography on a 12g silica gel cartridge, using gradient elution with 0-100% of ethyl acetate in hexane. Pure fractions were collected and concentrated to afford 44 mg of the desired product N-[(2,4-dichlorophenyl)methyl]-4-methanesulfonamidobenzamide (16.0 % yield).

^1H NMR (300 MHz, DMSO-d6) δ ppm 3.07 (s, 3 H) 4.49 (d, J=5.46 Hz, 2 H) 7.26 (m, J=8.67 Hz, 2 H) 7.34 (d, J=8.67 Hz, 1 H) 7.41 (d, J=8.10 Hz, 1 H) 7.62 (s, 1 H) 7.89 (m, J=8.48 Hz, 2 H) 8.99 (br. s., 1 H) 10.15 (s, 1 H)

^13C NMR (75 MHz, DMSO-d6) δ ppm 39.68, 40.24, 118.01, 127.32, 128.57, 128.66, 128.81, 130.01, 132.17, 132.88, 135.73, 141.46,165.89

LCMS predicted: m/z: m/z: 372.01 (100.0%), 374.01 (69.3%), 373.01 (17.9%), 376.00 (13.1%), 375.01 (11.8%), 377.01 (2.3%), 376.01 (1.5%), 374.02 (1.3%) seen: [M+H]^+ (major) 373.0, 374.9 also [M+CH₃CN+H]^+ (major) 413.9, 415.9
Scheme 2: Synthesis of ML402

**Step 1: Preparation of 2-[2-(4-chloro-2-methylphenoxy)ethyl]-2,3-dihydro-1H-isoindole-1,3-dione**

The preparation of this known compound was based on that described. A solution of diethyl azodicarboxylate (3.583 ml, 7.8 mmol, 1.5 equiv.) in dry THF (25 ml) was added under dry nitrogen to a solution of 2-(4-chlorophenoxy) ethylamine (1.0 g, 5.2 mmol, 1.0 equiv.), triphenylphosphine (2.06 g, 7.8 mmol, 1.5 equiv.) and 4-chloro-2-methylphenol (1.19 g, 7.8 mmol, 1.5 equiv.) in dry THF (50 ml). The reaction mixture was stirred at room temperature overnight then concentrated. The residue was re-suspended in diethyl ether and the solid filtered off on Celite. The filtrate was concentrated and the residue was re-dissolved in dichloromethane and then purified by silica gel chromatography on an 80 g silica gel cartridge using gradient elution with 0-20% ethyl acetate in hexane to afford 0.92 g of the desired product 2-[2-(4-chloro-2-methylphenoxy)ethyl]-2,3-dihydro-1H-isoindole-1,3-dione (56 % yield).

**Step 2: Preparation of 1-(2-aminoethoxy)-4-chloro-2-methylbenzene**

Deprotection of the amine function was accomplished according to the procedure described. A 50 ml reaction bottle with a magnetic stir bar was charged with 2-[2-(4-chloro-2-methylphenoxy)ethyl]-2,3-dihydro-1H-isoindole-1,3-dione (417 mg, 1.3 mmol, 1.0 equiv.) and methanol (20 ml) was added, followed by hydrazine monohydrate (500 µL, 10.3 mmol, 7.8 equiv.). The reaction mixture was stirred for 72 hours at room temperature after which time the reaction was judged complete. The solvent was evaporated chloroform was added, the organic layer was washed with water and brine and then dried over Na₂SO₄, decanted, and concentrated. The residue was re-suspended in dichloromethane and applied purified by silica gel chromatography on a 12g silica gel cartridge using gradient elution 1-10% of 1% NH₄OH in methanol in dichloromethane to afford 79 mg of the desired product 1-(2-aminoethoxy)-4-chloro-2-methylbenzene (32 % yield).
**Step 3: Preparation of N-[2-(4-chloro-2-methylphenoxy)ethyl]thiophene-2-carboxamide (ML402)**

A 50 ml reaction bottle with a magnetic stirbar was charged with 1-(2-aminoethoxy)-4-chloro-2-methylbenzene (39 mg, 0.2 mmol, 1.0 equiv.), thiophene-2-carboxylic acid (27 mg, 0.2 mmol, 1.0 equiv.) and N,N-dimethylformamide (2.000 mL, 25.9 mmol, 122.4 equiv.). To the mixture was added HATU (121 mg, 0.3 mmol, 1.5 equiv.) followed by triethylamine (89 µL, 0.6 mmol, 3.0 equiv.) The reaction mixture was stirred for 3 hours at room temperature and then partitioned between ethyl acetate and 10% aqueous citric acid. The organic layer was separated and washed with brine, then dried over Na₂SO₄, decanted, and concentrated. The residue was re-suspended in dichloromethane and purified by automated silica gel flash chromatography on a 12g silica gel cartridge, using gradient elution with 0-100% of ethyl acetate in hexane. The resulting residue (18.5mg) was further purified by preparative HPLC (C18 column, 40% to 90% methanol in water, 0.05% formic acid) to afford 12 mg of the desired product N-[2-(4-chloro-2-methylphenoxy)ethyl]thiophene-2-carboxamide (19 % yield).

**1H NMR (300 MHz, CHLOROFORM-d)** δ ppm 2.24 (s, 3 H) 3.89 (q, J=5.27 Hz, 2 H) 4.14 (t, J=5.50 Hz, 2 H) 6.48 (br. s., 1 H) 6.76 (d, J=8.10 Hz, 1 H) 7.06 - 7.17 (m, 3 H) 7.51 (d, J=5.09 Hz, 1 H) 7.54 (d, J=3.58 Hz, 1 H)

**13C NMR (75 MHz, CHLOROFORM-d)** δ ppm 16.18, 39.39, 67.08, 112.30, 125.66, 126.52, 127.69, 128.25, 128.49, 130.06, 130.54, 138.45, 155.08, 161.95

LCMS predicted: m/z: 295.04 (100.0%), 297.04 (36.5%), 296.05 (15.4%), 298.04 (5.9%), 299.04 (1.7%), 297.05 (1.6%), 296.04 (1.2%) seen: [M+H]⁺ (major) 296.1, 298.1
Scheme 3: Synthesis of ML335a

Step 1: Preparation of trans tert-butyl N-(4-{[(2,4-dichlorophenyl)methyl]carbamoyl}cyclohexyl)carbamate

A round bottom flask was charged with Boc-trans-1,4-aminocyclohexane carboxylic acid (181 mg, 0.7 mmol, 1.0 equiv.) and N,N-dimethylformamide (5 mL). To the mixture was added HATU (424 mg, 1.1 mmol, 1.5 equiv.) followed by 2,4-dichlorobenzylamine (100 µL, 0.7 mmol, 1.0 equiv.), and finally triethylamine (0.31 mL, 2.2 mmol, 3.0 equiv.). The reaction mixture was stirred for an hour at room temperature and then diluted with ethyl acetate and 10% aqueous citric acid. The organic layer was separated and washed with brine, leading to the formation of a precipitate. The entire two-phase mixture was therefore filtered and the precipitate collected on a paper filter. This afforded the product, trans tert-butyl N-(4-{[(2,4-dichlorophenyl)methyl]carbamoyl}cyclohexyl)carbamate (257 mg, 0.6 mmol, 86%), which was used in the next step without further purification.

1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.10 (dd, J=11.87, 3.39 Hz, 2 H) 1.44 (s, 8 H) 1.57 (dd, J=12.34, 3.30 Hz, 2 H) 1.93 (d, J=13.38 Hz, 2 H) 1.98 - 2.18 (m, 3 H) 4.47 (d, J=6.03 Hz, 2 H) 5.93 (br. s., 1 H) 7.21 (dd, J=8.19, 1.98 Hz, 1 H) 7.31 (d, J=8.29 Hz, 1 H) 7.38 (d, J=2.07 Hz, 1 H) LCMS: predicted: m/z: 400.13 (100.0%), 402.13 (64.1%), 401.14 (21.0%), 403.13 (13.7%), 404.13 (10.7%), 402.14 (2.7%), 405.13 (2.2%), 404.14 (1.3%) Seen: (only fragment, m-15 observed (major): 386.1, 388.1

Step 2: Preparation of trans-4-amino-N-[[2,4-dichlorophenyl]methyl]cyclohexane-1-carboxamide hydrochloride

A 25 mL round-bottom flask was charged with trans tert-butyl N-[[2,4-dichlorophenyl]methyl]carbamoyl)cyclohexyl)carbamate (257 mg, 0.6 mmol) dissolved in dichloromethane (4.0 ml) with stirring, and then treated with a hydrochloric acid solution (4.0 ml of 4M
solution in 1,4 dioxane, 16.0 mmol, 25.0 equiv.). The reaction mixture was stirred overnight and then the solvent was evaporated to afford crude trans-4-amino-N-[(2,4-dichlorophenyl)methyl]cyclohexane-1-carboxamide hydrochloride (244 mg, 0.7 mmol), which was used in the next step without further purification.

1H NMR (300 MHz, DMSO-d6) δ ppm 1.26 - 1.57 (m, 4 H) 1.83 (d, J=12.06 Hz, 2 H) 2.00 (d, J=9.61 Hz, 2 H) 2.19 (m, 1 H) 2.95 (br. s., 1 H) 4.27 (d, J=5.27 Hz, 1 H) 7.30 (d, J=8.29 Hz, 1 H) 7.42 (d, J=8.85 Hz, 1 H) 7.61 (s, 1 H) 8.15 (br. s., 2 H) 8.46 (t, J=5.5 Hz, 1 H)

LCMS, predicted: m/z: 300.08 (100.0%), 302.08 (64.2%), 301.08 (15.9%), 304.07 (10.2%), 303.08 (9.8%), 305.08 (1.6%), 302.09 (1.1%) seen: [M+H]+ (major) = 300.89, 302.91

**Step 3: Preparation of trans-N-[(2,4-dichlorophenyl)methyl]-4-ethenesulfonamidocyclohexane-1-carboxamide**

A 25 mL round bottom flask was charged with trans-4-amino-N-[(2,4-dichlorophenyl)methyl]cyclohexane-1-carboxamide hydrochloride (50 mg, 0.1 mmol, 1.3 equiv.) dissolved in dichloromethane (2.5 ml). The mixture was stirred in an ice bath and treated with triethylamine (0.083 ml, 0.6 mmol, 5.3 equiv.), followed by methanesulfonyl chloride (9 µl, 0.1 mmol, 1.0 equiv.). The reaction mixture was stirred for 3 hours while warming to room temperature. During the reaction a precipitate formed, and this was collected by vacuum filtration on a paper filter. The solid was saved and the filtrate was applied directly on a 12g silica gel cartridge and purified using gradient elution (0-100% ethyl acetate in hexanes). Relevant fractions were collected and concentrated to afford 19.5 mg of the product, which was identical to the precipitate collected directly from the reaction mixture. Combined, this amounted to 39 mg of the desired product, trans-N-[(2,4-dichlorophenyl)methyl]-4-ethenesulfonamidocyclohexane-1-carboxamide (0.1 mmol, 93% over two steps).

1H NMR (300 MHz, DMSO-d6) δ ppm 1.20 (m, 2 H) 1.43 (m 2 H) 1.76 (d, J=10.36 Hz, 2 H) 1.92 (d, J=9.23 Hz, 2 H) 2.03-2.20 (m, 1 H) 2.89 (s, 3 H) 2.98 - 3.16 (m, 1 H) 4.25 (d, J=5.84 Hz, 2 H) 6.98 (d, J=7.54 Hz, 1 H) 7.26 (d, J=8.10 Hz, 1 H) 7.41 (dd, J=8.38, 2.17Hz, 1 H) 7.59 (d, J=2.07 Hz, 1 H) 8.29 (t, J=5.50 Hz, 1 H)

13C NMR (100 MHz, DMSO-d6) δ ppm 28.19, 32.92, 39.38, 41.00, 42.70, 51.49, 127.30, 128.53, 129.91, 132.08, 132.89, 135.77, 174.92

LCMS: Predicted: m/z: 378.06 (100.0%), 380.05 (68.4%), 379.06 (17.4%), 382.05 (13.2%), 381.06 (11.5%), 383.05 (2.3%), 380.06 (2.1%), 382.06 (1.4%), 381.05 (1.0%), observed (m/z): [M+H]+ = (major) 379.1, 381.1
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ML335-\(^1\text{H}\)

ML335-\(^{13}\text{C}\)
ML335-$^{13}$C

**solvent region**

ML335-$^{13}$C

**aryl region**
ML402-^{13}C
alkyl region

ML402-^{13}C
aryl region
ML335a-\textsuperscript{13}C

alkyl region

ML335a-\textsuperscript{13}C

aryl region