Unexpected link between an antibiotic, pannexin channels and apoptosis

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Plasma membrane pannexin 1 channels (PANX1) release nucleotide find-me signals from apoptotic cells to attract phagocytes. Here we show that the quinolone antibiotic trovafloxacin is a novel PANX1 inhibitor, by using a small–molecule screen. Although quinolones are widely used to treat bacterial infections, some quinolones have unexplained side effects, including deaths among children. PANX1 is a direct target of trovafloxacin at drug concentrations seen in human plasma, and its inhibition led to dysregulated fragmentation of apoptotic cells. Genetic loss of PANX1 phenocopied trovafloxacin effects, revealing a non-redundant role for pannexin channels in regulating cellular disassembly during apoptosis. Increase in drug-resistant bacteria worldwide and the dearth of new antibiotics is a major human health challenge. Comparing different quinolone antibiotics suggests that certain structural features may contribute to PANX1 blockade. These data identify a novel linkage between an antibiotic, pannexin channels and cellular integrity, and suggest that re-engineering certain quinolones might help develop newer antibacterials.

Pannexins are four-pass transmembrane channels identified as a new family of channels for small molecules (up to ~1 kDa) across the plasma membrane 12–15 (Fig. 1a). Among the three vertebrate pannexin family members (PANX1, PANX2 and PANX3), PANX1 is the most widely expressed 1 and is implicated in regulating neutrophil activation 1, airway inflammation 6, human immunodeficiency virus infection 7, vasconstriction 8, migraine 9 and other neurological disorders 10. This broad and diverse range of functions may in part arise from pannexin channel-mediated release of purines such as ATP into the extracellular space, where purinergic signalling can influence multiple physiological processes 11. Thus, PANX1 is an attractive therapeutic target for human diseases and we sought to identify small molecules that can modulate PANX1 function.

Trovafoxacin inhibits PANX1 channel activity

Caspe-mediated cleavage of PANX1 carboxy terminus during apoptosis leads to PANX1 channel opening and release of nucleotide find-me signals from early apoptotic cells to recruit phagocytes 12–15 (Fig. 1a). This channel opening also allows the entry of fluorescent dyes, including TO-PRO-3 11,13 (Fig. 1a). We optimized TO-PRO uptake by apoptotic Jurkat cells as a reliable, medium-throughput, flow-cytometry-based assay for monitoring PANX1 activity. We tested a 'library of pharmacologically active compounds' (LOPAC 1,280) containing 1,280 small molecules targeting a diverse range of cellular processes—including currently marketed drugs, failed candidates, and bioactive molecules with known activities. The initial screen revealed three potential PANX1 inhibitors that were tested in secondary screens. Among them, trovafloxacin (a quinolone-based antibiotic) was identified as a potent inhibitor of TO-PRO-3 uptake by apoptotic cells (Fig. 1b). The use of trovafloxacin in patients has been linked to serious adverse side effects, including effects on the central nervous system, hepatic toxicity and in some cases mortality, but the molecular target(s) of trovafloxacin in mammalian cells is unclear 14–17. Trovafloxacin inhibition of PANX1 was dose-dependent, and comparable to the known pannexin inhibitor carbamoxalone (CBX) (Fig. 1c). Trovafloxacin also inhibited ATP release from apoptotic cells (Fig. 1d). Importantly, trovafloxacin did not inhibit caspase 3/7 activation, or caspase-mediated PANX1 cleavage during apoptosis (Extended Data Fig. 1a, b), ruling these out as reasons.

Several additional analyses suggested trovafloxacin could directly target PANX1 channel activity. Adding trovafloxacin to cells already undergoing apoptosis (that is, with open PANX1 channels) acutely blocked TO-PRO-3 uptake (Extended Data Fig. 1c, d). When we measured apoptosis-induced plasma membrane PANX1 currents at the single-cell level, by whole-cell patch-clamp recordings, trovafloxacin rapidly inhibited the inward current (at ~50 mV), with minimal effect on outward current (at ~80 mV) (Fig. 1e and Extended Data Fig. 1e). We have previously shown that the C-terminal tail of PANX1 blocks the channel pore, and that adding excess soluble C-terminal tails can inhibit ‘open’ PANX1 channels, especially the inward current ( analogous to trovafloxacin) 14. In contrast, CBX blocked both inward and outward currents 11,13,19 (Fig. 1e, f). Trovafloxacin did not inhibit connexin 43 gap junction or PANX2 (Extended Data Fig. 1f–i). Using a tobacco etch virus (TEV)-protease system to cleave the C-terminal tail of recombinant PANX1 and induce channel activity (independent of apoptosis) 14, trovafloxacin again potently blocked open PANX1 channels (Fig. 1g). To test direct channel blocking, we recorded TEV-cleaved PANX1 single-channel activity in excised inside-out patch-clamp by adding trovafloxacin to the patch; this led to an increase in the time spent in the closed state, with open probability (NPO) of ~0.85 in control conditions reduced to ~0.15 with trovafloxacin (Fig. 1h). The half maximal inhibitory concentration (IC50) of trovafloxacin was ~4 µM for the PANX1 inward current (Fig. 1i), similar to concentrations normally achieved in human plasma (2–10 µM) 20,21. These data indicated that mammalian PANX1 channels could be a direct target of antibiotic trovafloxacin.

Trovafoxacin promotes fragmentation of apoptotic cells

Next, we investigated trovafloxacin effects on apoptotic cells via microscopy and made several surprising observations. Apart from reducing
PTOSIS, because the human body turns over more than 200 billion cells through a process known as cellular disassembly. Although trovafloxacin itself did not induce apoptosis in a concentration-dependent manner (Fig. 2c, d). Instead, apoptosis was promoted formation of apoptotic bodies (Fig. 2e, f). Second, this effect was not dependent on the mode of apoptosis induction, as blocking PANX1 during Fas- or ultraviolet-mediated apoptosis promoted formation of apoptotic bodies (Fig. 2g).

**PANX1 regulates the disassembly of apoptotic cells**

Although some studies have addressed apoptotic cell disassembly, the mechanisms that control the formation of apoptotic bodies are not well understood. However, in these experiments we could not track thymic trovafloxacin concentrations, and trovafloxacin in vivo could also have had other effects beyond PANX1. Therefore, we generated mice carrying deletion of CBX-sensitive current and TO-PRO-3 uptake (Extended Data Fig. 4a) resulted in increased formation of apoptotic bodies (Fig. 2h).

**Trovafloxacin and PANX1 regulate apoptotic cell disassembly in vivo**

To address how trovafloxacin and PANX1 might affect apoptosis in a whole animal, we used intraperitoneal dexamethasone injection to induce synchronous apoptosis of a large fraction of mouse thymocytes in vivo (Fig. 3c). Administering trovafloxacin with dexamethasone promoted the fragmentation of the apoptotic thymocytes (Fig. 3d, with fewer SSChigh particles; see Extended Data Figs 5 and 6 for gating strategy). However, in these experiments we could not track thymic trovafloxacin concentrations, and trovafloxacin in vivo could also have had other effects beyond PANX1. Therefore, we generated mice carrying deletion of CBX-sensitive current and TO-PRO-3 uptake (Extended Data Fig. 4a) resulted in increased formation of apoptotic bodies (Fig. 2h).
of Panx1, using embryonic stem cells engineered to disrupt the Panx1 gene (Extended Data Fig. 7). In the thymus of Panx1−/− and Panx1+/− mice treated with dexamethasone, the fragmentation of apoptotic cells was enhanced (Fig. 3e), essentially phenocopying the effect of trovafloxacin treatment in wild-type mice (Fig. 3d). The apoptotic particles from Panx1−/− and Panx1+/− mice also showed reduced TO-PRO-3 uptake (Fig. 3e). To rule out that loss of PANX1 in other tissues contributed to the observed thymic phenotypes in the global knockout mice, we specifically deleted Panx1 in thymocytes by crossing the Panx1fl/fl mice with Lck-Cre mice, where Cre is expressed in the T-cell lineage (Extended Data Fig. 7). Similar apoptotic fragmentation and loss of TO-PRO-3 uptake were observed when PANX1 was deleted specifically in thymocytes (Fig. 3f), indicating a cell-autonomous effect. Ex vivo, apoptotic thymocytes from Panx1 global knockout mice or the PANX1 WT transfected cells expressing Panx1DN mutant, but decreased in PANX1 WT transfected cells (n = 3). The PANX1 WT effect is reversed by CBX. Error bars represent s.e.m.

**Figure 2** | Trovafloxacin-mediated inhibition of PANX1 promotes formation of smaller apoptotic bodies. a, Schematic for apoptosis progression and generation of apoptotic bodies, derived from multi-parameter time-lapse analyses of Jurkat cells. b, Images of vehicle- or trovafloxacin-treated apoptotic cells stained with annexin V or TO-PRO-3. Note the annexin V−/TO-PRO-3− apoptotic bodies (~0.9–5 μm) after trovafloxacin treatment. c, Trovafloxacin increases particles with reduced size (FSC) and complexity (SSC). d, Generation of apoptotic bodies is enhanced by trovafloxacin, but not levofloxacin and ciprofloxacin. e, Right, percentage of fragmented cell bodies (Extended Data Fig. 3f). Thus, the extracellular ATP levels and P2 purinergic receptor signalling are probably not directly involved in regulating apoptotic cell integrity.

**PANX1 regulates formation of string-like apoptopodia**

We next used time-lapse microscopy to monitor cell morphology and TO-PRO-3 uptake. In our experimental conditions with Jurkat cells induced to undergo Fas-mediated apoptosis, most cells begin to show rounding and membrane blebs around 60–90 min, and continue to bleed for another 30–60 min, a time period referred here as ‘dynamic blebbing’. Remarkably, very few of these blebs detach from the apoptotic cell, and after cessation of the dynamic blebbing, these blebs remain attached to the dying cell (Fig. 4a, b and Supplementary Video 1). When we added TO-PRO-3 into the medium and tracked its uptake as a measure of PANX1 activity, TO-PRO-3 staining was detected before cell rounding and the onset of blebbing (Fig. 4c and Extended Data Fig. 4c). This indicates that PANX1 channels are activated/opened before the appearance of apoptotic cell morphology.

One possible mechanism for this increased cellular fragmentation is the lack of ATP that would normally be released by apoptotic cells, and the various autocrine and paracrine signalling via P2 purinergic receptors. However, adding exogenous ATP to apoptotic cells when Panx1 channel activity was blocked did not inhibit apoptotic bodies formation (Extended Data Fig. 3c, d). Conversely, adding recombiant apyrase (to hydrolyse ATP) during induction of apoptosis did not promote the formation of apoptotic bodies (Extended Data Fig. 3e). Furthermore, treatment with suramin, a broad inhibitor of P2 family G-protein-coupled receptors, did not enhance formation of apoptotic bodies (Extended Data Fig. 3f). Thus, the extracellular ATP levels and P2 purinergic receptor signalling are probably not directly involved in regulating apoptotic cell integrity.

**Figure 3** | Trovafloxacin increases apoptotic cell fragmentation. a, Jurkat cells (Annexin V+), apoptotic cell bodies (Annexin V−). b, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy) (Panx1DN) relative to controls (Panx1WT). c, Time course of apoptotic cell death (Annexin V+). g, Time course of apoptotic cell death (Annexin V−). h, representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). i, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). j, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). k, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). l, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). m, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). n, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). o, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). p, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). q, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). r, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). s, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). t, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). u, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). v, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). w, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). x, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). y, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy). z, Representative 4 h time-lapse images monitoring apoptotic cell morphology and apoptotic bodies formation index (via microscopy).
Trovafloxacin in thymic apoptosis

Particles (blebbing was blocked by inhibiting actomyosin contraction or the Rho-kinase to downregulate the formation of apoptopodia. Interestingly, when activity is probably continuously required during apoptosis progressing were linked. PANX1-mediated TO-PRO-3 uptake began before apoptosis induction. These membrane protrusions after apoptosis induction. These apoptopodia (Extended Data Fig. 8d–f). These data indicate that the apoptopodia that arise owing to PANX1 inhibition are specifically, pannexin channel function is required to restrain detachment and detachment of surfaces (Extended Data Fig. 10).

Trovaflonax-mediated enhancement of apoptotic bodies formation (Fig. 3j). Fas-, dexamethasone- or ultraviolet-induced apoptosis was blocked by inhibiting blebbing reduced the formation of apoptotic bodies in Jurkat cells with PANX1 function disrupted by pharmacological block, or in

PANX1-deficient thymocytes (Extended Data Fig. 8d–f). These data indicate that the apoptosis that arise owing to PANX1 inhibition are independent of blebbing, but that the annexin V+ vesicular structures seen at the end of apoptosis are dependent on blebbing and contribute to apoptotic bodies formation. LR73 fibroblasts induced to undergo apoptosis also showed the formation of apoptosis and apoptotic bodies when PANX1 function was impaired (Extended Data Fig. 9). Thus, trovafloxacin-mediated enhancement of apoptotic bodies formation helped uncover a new step during the early stages of apoptosis; specifically, pannexin channel function is required to restrain detachment of blebs from the apoptotic cells, and in turn, regulate the nature of apoptotic cell disassembly (Extended Data Fig. 10).

PANX1 inhibition varies among different quinolones

Although quinolone antibiotics are effective at killing bacteria by targeting their topoisomerases, and are used worldwide to treat various bacterial infections, some quinolones have serious side effects (incidence up to 5%) in the liver, skin, tendon, gastrointestinal tract, central nervous system and cardiovascular system that are not readily explained.

**Figure 3** | **Panx1** regulates disassembly of apoptotic thymocytes.

**a, b.** Increase in formation of apoptotic bodies (a), or decrease in SSChigh particles (b) from dexamethasone (Dex)-treated thymocytes ex vivo by trovafloxacin, CBX, and probenecid (n = 3). c. Schematic for testing trovafloxacin in thymic apoptosis in vivo. d. Reduction in SSChigh apoptotic bodies in thymi of wild-type mice treated with Dex + trovafloxacin (n = 6). e, f. Complexity and TO-PRO-3 uptake (fold change relative to untreated) in thymi of indicated mice injected intraperitoneally with Dex (n = 10 for Panx1+/+, n = 8 for Panx1+/–, n = 7 for Panx1–/–, n = 8 for Panx1fl/fl and Lck-Cre/Panx1fl/fl). g, h. Formation of apoptotic bodies (upper) or TO-PRO-3 uptake (lower) ex vivo in Dex-treated thymocytes from Panx1+/+ or Lck-Cre/Panx1fl/fl thymocytes. i. Reduction in SSChigh subapoptotic particles in thymocytes from Panx1+/+ and Lck-Cre/Panx1fl/fl mice. (g–j, n = 6 for Panx1+/+, n = 8 for Panx1+/–, n = 6 for Panx1–/–, n = 4 for Panx1fl/fl, n = 3 for Lck-Cre/Panx1fl/fl.) j. Time-lapse images monitoring apoptotic cell morphology of thymocytes. Arrows, apoptopodia. Error bars represent s.e.m.

**Data in a, b, and j** are representative of at least two independent experiments. See Extended Data Figs 5 and 6 for gating strategy. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student’s two-tailed t-test.

**Figure 4** | **Panx1** regulates disassembly of apoptotic thymocytes.

**a–d.** Increase in formation of apoptotic bodies (a), or decrease in SSChigh particles (b) from dexamethasone (Dex)-treated thymocytes ex vivo by trovafloxacin, CBX, and probenecid (n = 3). c. Schematic for testing trovafloxacin in thymic apoptosis in vivo. d. Reduction in SSChigh apoptotic bodies in thymi of wild-type thymocytes treated with Dex + trovafloxacin (n = 6). e, f. Complexity and TO-PRO-3 uptake (fold change relative to untreated) in thymi of indicated mice injected intraperitoneally with Dex (n = 10 for Panx1+/+, n = 8 for Panx1+/–, n = 7 for Panx1–/–, n = 8 for Panx1fl/fl and Lck-Cre/Panx1fl/fl). g, h. Formation of apoptotic bodies (upper) or TO-PRO-3 uptake (lower) ex vivo in Dex-treated thymocytes from Panx1+/+ or Lck-Cre/Panx1fl/fl thymocytes. i. Reduction in SSChigh subapoptotic particles in thymocytes from Panx1+/+ and Lck-Cre/Panx1fl/fl mice. (g–j, n = 6 for Panx1+/+, n = 8 for Panx1+/–, n = 6 for Panx1–/–, n = 4 for Panx1fl/fl, n = 3 for Lck-Cre/Panx1fl/fl.) j. Time-lapse images monitoring apoptotic cell morphology of thymocytes. Arrows, apoptopodia. Error bars represent s.e.m.

**Data in a, b, and j** are representative of at least two independent experiments. See Extended Data Figs 5 and 6 for gating strategy. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student’s two-tailed t-test.

**Figure 5** | **Panx1** regulates disassembly of apoptotic thymocytes.

**a–d.** Increase in formation of apoptotic bodies (a), or decrease in SSChigh particles (b) from dexamethasone (Dex)-treated thymocytes ex vivo by trovafloxacin, CBX, and probenecid (n = 3). c. Schematic for testing trovafloxacin in thymic apoptosis in vivo. d. Reduction in SSChigh apoptotic bodies in thymi of wild-type thymocytes treated with Dex + trovafloxacin (n = 6). e, f. Complexity and TO-PRO-3 uptake (fold change relative to untreated) in thymi of indicated mice injected intraperitoneally with Dex (n = 10 for Panx1+/+, n = 8 for Panx1+/–, n = 7 for Panx1–/–, n = 8 for Panx1fl/fl and Lck-Cre/Panx1fl/fl). g, h. Formation of apoptotic bodies (upper) or TO-PRO-3 uptake (lower) ex vivo in Dex-treated thymocytes from Panx1+/+ or Lck-Cre/Panx1fl/fl thymocytes. i. Reduction in SSChigh subapoptotic particles in thymocytes from Panx1+/+ and Lck-Cre/Panx1fl/fl mice. (g–j, n = 6 for Panx1+/+, n = 8 for Panx1+/–, n = 6 for Panx1–/–, n = 4 for Panx1fl/fl, n = 3 for Lck-Cre/Panx1fl/fl.) j. Time-lapse images monitoring apoptotic cell morphology of thymocytes. Arrows, apoptopodia. Error bars represent s.e.m.

**Data in a, b, and j** are representative of at least two independent experiments. See Extended Data Figs 5 and 6 for gating strategy. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student’s two-tailed t-test.
Figure 4 | Formation of string-like apoptopodia after membrane blebbing correlates with formation of apoptotic bodies. a, Schematic of apoptotic Jurkat cells with normal or impaired PANX1, based on time-lapse microscopy. b, Time-lapse images monitoring progression of apoptotic cell morphology. c, PANX1-mediated TO-PRO-3 entry occurs before membrane blebbing as determined by quantifying TO-PRO-3 uptake by apoptotic cells \((n = 15)\) in time-lapse imaging (normalized to first sign of cell rounding). TO-PRO-3 uptake was undetectable in live cells \((n = 11)\). d, Trovafloxacin-treated apoptotic cells or expressing PANX1-DN mutant show formation of apoptopodia. e, Apoptopodia have membranes with exposed phosphatidylserine and apoptotic blebs at the end of the protrusion. 

Surprisingly, in contrast to trovafloxacin, two other structurally related quinolone antibiotics ciprofloxacin and levofloxacin did not block PANX1-dependent dye uptake (Fig. 1c), despite all three being equally effective in inhibiting bacterial growth (Extended Data Fig. 1j). Comparing the molecular structure of trovafloxacin with ciprofloxacin and levofloxacin suggested that certain features of trovafloxacin, such as the fluorinated ring at position N1 (Fig. 4h), might contribute to its effect on PANX1 (Figs 1c and 2d). We next asked whether other quinolones with a fluorinated ring at position N1 can inhibit PANX1, and tested difloxacin and tosufloxacin (Fig. 4h) that are currently available commercially in some Asian countries. Although less potent than trovafloxacin, both difloxacin and tosufloxacin partially inhibited ATP release and TO-PRO-3 uptake by apoptotic cells, and promoted the formation of apoptotic bodies (Fig. 4i). These data do not exclude other molecular features of trovafloxacin that may also contribute to PANX1 inhibition, such as the C7 position of the quinolone (Fig. 4h).

Discussion

The data presented here suggest an unexpected but intriguing link between the antibiotic trovafloxacin, eukaryotic pannexin channels,
and a specific step in progression through apoptosis. Specifically, they provide three key insights. First, trovafloxacin, originally marketed by Pfizer, showed tremendous promise, but was linked to severe toxicity and unexplained deaths among children in a trial in Nigeria, and was discontinued. Because billions of cells are turned over daily via caspase-mediated apoptosis in the human body as part of normal life, and trovafloxacin can directly inhibit the mammalian PANX1 channels at doses normally achieved in humans, this could, in part, provide a mechanism for trovafloxacin toxicity. Second, our data identify a new role for pan- nexin channels in maintaining cellular integrity during apoptosis. Fragmentation of apoptotic cells into smaller apoptotic bodies has been seen in vitro and in vivo, but the mechanism(s) regulating this disassembly process is not well understood. The trovafloxacin-mediated blockage of PANX1 uncovered a previously unappreciated step during apoptosis, and revealed an essential and non-redundant role for pan- nexin channels in the ordered disassembly of apoptotic cells. Third, the dearth of new antibiotics in the pipeline despite the steady increase in drug-resistant bacteria worldwide is a significant health threat. Interestingly, quinolone antibiotics such as ciprofloxacin and levofloxacin do not share the inhibitory effect of trovafloxacin towards the mammalian PANX1, and certain features in trovafloxacin might in part explain its toxicity. Further studies on the interaction of quinolones with PANX1 and the associated apoptosis pathways may provide clues to the underlying reasons for the idiosyncratic and irreversible liver toxicity observed with trovafloxacin. Unlocking such clues could re-invigorate industrial interest in the quinolone class for the production of safer, and more potent antibacterials.

METHODS SUMMARY

For drug screening targeting pannexin channels, Jurkat cells were induced to undergo apoptosis (4 h) by anti-Fas (250 ng/ml) in the presence of 10 μM of each compound from the LOPAC1280 library (Sigma-Aldrich). Cells were then stained with TO-PRO-3 and analysed by flow cytometry. Chimeric mice were generated using Panx1-targeted embryonic stem cells (from KOMP repository) and crossed with β-actin/Fp mice (JaxMice) to delete the neomycin cassette, and crossed with Elf-Cre mice (JaxMice) or Lck-Cre mice (JaxMice) to delete PANX1 globally or specifically in thymic macrophages for determining apoptotic cell fragmentation by flow cytometry. Jurkat cells were stained with annexin V-FLICA, 7-AAD and TO-PRO-3, whereas thymocytes from in vivo experiments were stained similarly with annexin V-FLICA, 7-AAD and TO-PRO-3. CD48 conjugated with phycoerythrin and CD44 conjugated with phycoerythrin-Cy7, with gating as per Extended Data Figs 2, 5 and 6. For determining apoptotic cell fragmentation by flow cytometry, membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. J Biol Chem 274, 21953–21962 (1999).

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Author Contributions I.K.H.P. designed, performed and analysed most of the experiments with input from K.S.R. and A.J.H.C. performed and analysed the patch-clamp studies with input from D.A.B. A.J.A. generated the PANX1 knockout mice and Jurkat cell lines expressing PANX1. J.M.K. helped with the drug screen and microscopy experiments. I.J.P. performed primary T-cell isolation and quantitative PCR. A.J.A. and I.K.H.P. carried out the initial characterization of PANX1-deficient mice. I.K.H.P. and K.S.R. wrote the manuscript with input from co-authors.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to K.S.R. (Rav@virginia.edu).
**METHODS**

**Reagents.** Library of Pharmacologically Active Compounds (LOPAC™; http://www.sigmaaldrich.com/chemistry/drug-discovery/validation-libraries/lopac1280-navigator.html), trovafloxacin, ciprofloxacin, levofloxacin, diflouxacin, tosufloxacin, carbadoxone, probenicid, blysibilitan, cytochalasin D, purified nucleotides, suramin and dexamethasone were obtained from Sigma-Aldrich, 7-AAD and TO-PRO-3 were purchased from Invitrogen. Annexin V-FITC, CD8α-PE (clone 53-6.7), CD4-PE-Cy7 (clone RM4-5) and anti-mouse CD16/CD32 (clone 93) were obtained from eBioscience. Other reagents were obtained as follows: anti-Fas (clone CH11, Millipore), z-VAD-FMK (Enzo Life Sciences), Q-VD-OPH (SM Biochemicals) and recombinant apyrase (New England Biolabs).

**Induction of apoptosis.** Jurkat cells in RPMI with 1% BSA were treated with 250 ng ml\(^{-1}\) anti-Fas (clone CH11) or 150 ml cm\(^{-2}\) ultraviolet C irradiation (Stratalinker). Primary thymocytes (collected from 5- to 7-week-old C57BL/6 mice, male and female) were treated with 50 μM dexamethasone for 5 h. LR73 cells were treated with 150 μM carbenoxolone. All treatments were incubated for indicated times at 37 °C, 5% CO\(_2\), unless noted otherwise.

**Drug screening for regulators of Panx1 function.** Jurkat cells (10⁶ cells ml\(^{-1}\) in DMEM +0.5% BSA) were induced to undergo apoptosis by anti-Fas treatment (250 ng ml\(^{-1}\)) in the presence of 10 μM of compounds in the LOPAC1280 (Sigma-Aldrich) for 4 h at 37 °C, 5% CO\(_2\). Cells were then stained with TO-PRO-3 (0.67 μM) for 10 min at room temperature and immediately placed on ice before analysis on a BD FACSCanto flow cytometer. The resultant flow cytometric data were analysed by FlowJo software (Tree Star).

**Determining cell viability by flow cytometry and ImageStream.** Samples were stained with annexin V-FITC, 7-AAD and TO-PRO-3 in annexin V binding buffer for 10 min at room temperature and immediately placed on ice before analysis on a BD FACSCanto flow cytometer, with the resultant flow cytometry data analysed by FlowJo software. Samples were also prepared for fixation and staining for immunohistochemistry on ImageStreamX Mark II (Amnis), with the resultant data analysed by IDEAS software.

**Generation of pannexin 1 knockout mice.** Panx1-targeted embryonic stem cells (EPD0309-3-B01) were obtained from the Knockout Mouse Project (KOMP) Repository. After blastocyst injections (performed by the transgenic core facility at the University of Virginia), chimerae were bred with C57BL/6 mice (JaxMouse). The resultant offspring were crossed with β-actin/Fli mice (JaxMouse) to delete the neo-cassette, thereby generating mice carrying a floxed Panx1 exon 3 allele (Panx1flo). (Extended Data Fig. 7). To generate Panx1 global knockout mice, Panx1flo mice were crossed with Ella-Cre mice (JaxMouse) expressing Cre from the two-cell stage of embryonic development to delete Panx1 exon 3 in all tissues. Remaining mice were crossed with C57BL/6 mice to remove Fli and Cre from the background. To generate mice that carry deletion of Panx1 specifically in thymocytes, Panx1flo mice were crossed with Lck-Cre mice (JaxMouse) expressing Cre under the Lck proximal promoter, which mediates deletion of Panx1 exon 3 from the double-negative stage of thymocyte development.

**Microscopy.** Live imaging was performed on a Zeiss microscope using a ×40 or ×60 oil immersion objective in a 37 °C/5% CO\(_2\) atmosphere. Jurkat cells at 5 × 10⁵ cells ml\(^{-1}\) in RPMI +1% BSA (600 μl) were seeded onto Lab-Tek II Chambered Coverglass (Nunc) by two successive centrifugations at 20g for 1 min before imaging. LR73 fibroblasts were cultured overnight on Chambered Coverglass before imaging. In certain experiments, TO-PRO-3 and annexin V-FITC were also present during the imaging process.

**Bacterial growth assay.** Overnight *Escherichia coli* culture was diluted 100-fold in LB medium before incubation with quinolones for 6 h. Bacterial growth was monitored by the absorbance at 600 nm using a FlexStation 3 plate reader, with the resultant data analysed using SoftMaxPro 5.4 software.

**Nucleotide measurement.** ATP in apoptotic cell supernatants was measured using a luciferase/luciferin assay (CellTiter-Glo; Promega) according to the manufacturer’s instructions.

**Caspase activation.** Caspase 3/7 activity assays were performed with the Caspase-Glo 3/7 (Promega) reagents, in accordance with the manufacturer’s instructions.

**Immunoblotting.** Samples were analysed by SDS-PAGE and immunoblotting using the following dilutions: anti-GFP (1:1,000; Santa Cruz), anti-ERK2 (1:3,000; Santa Cruz), anti-Caspase 3 (1:2,500; Santa Cruz) and affinity purified rabbit anti-mouse Panx1 (0.2 μg ml\(^{-1}\)).

**Patch-clamp analysis.** Electrophysiological recordings were made at room temperature using an Axopatch 200B amplifier (Molecular Devices). Whole-cell recordings were carried out in Jurkat cells or transiently transfected HEK293T cells with cDNAs of interest and primary thymocytes (RNeasy, Qiagen) using Superscript III (Invitrogen). Quantiative PCR was performed on the ABI StepOnePlus Real-time PCR instrument with TaqMan probes (Applied Biosystems). Levels of mouse Panx1 mRNA were normalized to *Gapdh*. TaqMan probes used were: mouse Panx1 (Mm00450900_m1) and mouse *Gapdh* (Mm03924291_m1).

**DNA fragmentation assay.** DNA laddering during apoptosis was characterized by agarose gel electrophoresis. In brief, 60,000 Jurkat cells were induced to undergo apoptosis by anti-Fas treatment for 2 h and lysed in TES lysis buffer containing RNase for 2 h at 37 °C. Cell lysates were subsequently treated with proteinase K for 8 h at 50 °C and DNA fragmentation was visualized by agarose gel electrophoresis.

**Quantitative PCR.** cDNA was synthesized from 50 ng of RNA isolated from primary thymocytes (RNeasy, Qiagen) using Superscript III (Invitrogen). Quantitative PCR was performed on the ABI StepOnePlus Real-time PCR instrument with TaqMan probes (Applied Biosystems). Levels of mouse Panx1 mRNA were normalized to *Gapdh*. TaqMan probes used were: mouse Panx1 (Mm00450900_m1) and mouse *Gapdh* (Mm03924291_m1).

**In vivo model of apoptosis.** 5-week-old C57BL/6 mice (male and female) were injected intraperitoneally with 12.5 mg kg\(^{-1}\) dexamethasone. Trovafloxacin (60 mg kg\(^{-1}\); intraperitoneally) was administrated 10 min prior to dex injection (12.5 mg kg\(^{-1}\); intraperitoneally), and trovafloxacin was also given at 2 and 4 h and the analysis performed at 6 h. Thymocytes were collected 6 h post dexamethasone injection, stained with annexin V-FITC, 7-AAD, TO-PRO-3, CD8α-PE and CD4-PE-Cy7, and analysed on a BD FACSCanto flow cytometer. The University of Virginia Animal Care and Use Committee approved all animal experiments.

**Statistical analyses.** Data are presented as means ± s.e.m. Statistical significance for comparisons was determined by unpaired Student’s two-tailed t-test. A P value less than 0.05 was considered statistically significant.

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Extended Data Figure 1 | Trovafloxacin does not block caspase activation or inhibit connexin 43 (Cx43) or pannexin 2 (Panx2) membrane currents.

a. Caspase 3/7 activation in Jurkat cells undergoing apoptosis is not altered by treatment with trovafloxacin (40 μM) (n = 3).
b. Proteolytic cleavage of PANX1–GFP during apoptosis is not inhibited by trovafloxacin (40 μM) or CBX (500 μM) treatment.
c. Schematic diagram for the acute treatment of apoptotic cells with trovafloxacin or CBX.
d. Acute trovafloxacin treatment inhibits TO-PRO-3 uptake by apoptotic Jurkat cells. Left, histograms showing TO-PRO-3 uptake by viable cells, apoptotic cells, or apoptotic cells treated with trovafloxacin or CBX (500 μM) post induction of apoptosis and analysed by flow cytometry. Right, uptake of TO-PRO-3 presented as (median fluorescence intensity, MFI) of viable cells or apoptotic cells (n = 3).
e. Inhibition of CBX-sensitive current in apoptotic cells treated with trovafloxacin (20 μM), as measured by whole-cell patch-clamp recording (n = 7).
f. Patch-clamp recordings from HEK293T cells expressing Cx43 and receiving indicated treatments. Whole-cell current at +80 mV is shown under conditions when bath solution was perfused with trovafloxacin (20 μM, blue shading) or gadolinium (Gd3+)(100 μM, pink shading). g. Current-voltage relationships of Cx43 current in HEK293T cells treated with or without trovafloxacin (20 μM) or Gd3+ (100 μM), with the current measured over a range of voltages. Exemplar traces in f and g are representative of 14 cells per group.
h. Patch-clamp recordings from HEK293T cells expressing mouse Panx2 and receiving indicated treatments. Whole-cell current at +80 mV is shown under conditions when bath solution was perfused with trovafloxacin (20 μM, blue shading) or carbenoxolone (CBX) (50 μM, pink shading). i. Current-voltage relationships of Panx2 current in HEK293T cells treated with or without trovafloxacin (20 μM) or CBX (50 μM), with the current measured over a range of voltages. Exemplar traces in h and i are representative of 4 cells per group.
j. Trovafloxacin, ciprofloxacin and levofloxacin inhibit bacterial growth. *Escherichia coli* growth (as measured by absorbance at 600 nm) in the presence of indicated concentrations of quinolones (n = 3). Error bars represent s.e.m.
Extended Data Figure 2 | Electronic gating strategy for the separation of different cellular and subcellular population of Jurkat cells undergoing apoptosis in vitro. a, Flow cytometric analysis showing each type of particles gated (see b below) has a distinctive level of cellular complexity (side scatter, SSC), cell size (forward scatter, FSC) as well as TO-PRO-3 (indicative of caspase-mediated activation of pannexin 1 channels), 7-AAD (indicative of membrane integrity) and annexin V (indicative of phosphatidylserine exposure) staining. b, Flow cytometry gating strategy used to distinguish viable cells, annexin V<sup>−</sup> apoptotic cells, annexin V<sup>+</sup> apoptotic cells, annexin V<sup>−</sup> particles, and apoptotic bodies. c, ImageStream analysis of particles gated using the same strategy as described in b. Representative images for each type of particles are shown. Jurkat cells were induced to undergo apoptosis by anti-Fas treatment (2 h) in all indicated experiments.
Extended Data Figure 3 | Inhibition of pannexin 1 promotes the formation of apoptotic bodies via a mechanism independent of extracellular ATP. 

a, CBX and probenecid enhance the generation of apoptotic bodies from cells undergoing ultraviolet-induced apoptosis (n = 3). 

b, Formation of apoptotic bodies after treatment with the indicated concentrations of CBX (n = 3). 

The corresponding TO-PRO-3 uptake by annexin V+ apoptotic cells at each CBX concentration is shown above the respective bars. 

c, d, Addition of exogenous ATP during apoptosis induction does not inhibit formation of apoptotic bodies in CBX-treated cells (n = 3) (c) or cells stably expressing the dominant-negative PANX1 mutant (PANX1 DN mutant) (n = 3) (d). 

e, Removal of extracellular ATP by apyrase does not promote formation of apoptotic bodies (n = 3). 

f, P2Y receptor antagonist suramin does not promote formation of apoptotic bodies (n = 3). Jurkat cells were induced to undergo apoptosis by anti-Fas treatment in all indicated experiments. Error bars represent s.e.m.
Extended Data Figure 4 | Pannexin 1 activity does not affect DNA fragmentation during apoptosis. a, b, TO-PRO-3 dye uptake (n = 3) (a) and DNA fragmentation (b) were assessed in Jurkat cells stably expressing the control vector, the dominant-negative PANX1 mutant (PANX1 DN mutant) or wild-type PANX1 (PANX1 WT). DNA fragmentation from cells induced to undergo apoptosis and treated with or without 500 μM CBX is also shown in b. c, Time-lapse images monitoring TO-PRO-3 dye uptake during progression of apoptosis in Jurkat cells with normal PANX1 function show that TO-PRO-3 uptake occurs before initiation of membrane blebbing. Jurkat cells were induced to undergo apoptosis by anti-Fas treatment (2 h). Error bars in a represent s.e.m.
Extended Data Figure 5 | Electronic gating strategy for the separation of different cellular and subcellular populations of primary thymocytes undergoing apoptosis ex vivo. 

**a,** Flow cytometry analysis showing each type of particle gated according to **b** has a distinctive level of SSC, FSC as well as TO-PRO-3 and annexin V staining. 

**b,** Flow cytometry analysis showing electronic gating strategy used to distinguish viable cells, annexin V$^-$ apoptotic cells, annexin V$^+$ apoptotic cells, and apoptotic bodies. 

**c,** ImageStream analysis of particles gated using the same strategy as described in **b**. Representative images for each type of particle are shown. Primary mouse thymocytes were induced to undergo apoptosis by dexamethasone (Dex) treatment in all indicated experiments.
Extended Data Figure 6 | Electronic gating strategy for analysing the complexity of subcellular apoptotic particles generated ex vivo and in vivo.

**a**, Flow cytometry analysis showing electronic gating strategy used to distinguish annexin V<sup>high</sup>, 7-AAD<sup>low</sup> subcellular particles generated from primary mouse thymocytes induced to undergo apoptosis via dexamethasone treatment. Subcellular apoptotic particles with high complexity (SSC high) or low complexity (SSC low) are gated as shown.

**b**, Flow cytometry analysis showing electronic gating strategy used to distinguish different subsets of apoptotic cell-derived particles generated in the thymus of mice injected intraperitoneally with dexamethasone (6 h). Annexin V<sup>high</sup>, 7-AAD<sup>low</sup>, CD4/CD8<sub>intermediate</sub> particles were initially selected and subsequently gated based on forward scatter (FSC, indicative of cell size). Apoptotic particles of interest (as indicated) are therefore defined as annexin V<sup>high</sup>, 7-AAD<sup>low</sup>, CD4/CD8<sub>intermediate</sub> and FSC<sub>low/intermediate</sub>.
Extended Data Figure 7 | Generation of conditional and global pannexin 1-deficient mice. a, Strategy for deletion of neomycin cassette and exon 3 of Panx1. b, Identification of mice with floxed Panx1 loci, assessed by PCR. c, mRNA levels of Panx1 in CD4^+ thymocytes relative to Gapdh. n = 3 mice per group. d, Immunoblotting of lysates from thymocytes with the indicated genotypes. e, Identification of mice with wild-type, heterozygous and homozygous Panx1-targeted loci, assessed by PCR. f, mRNA levels of Panx1 in thymocytes with the indicated genotypes relative to Gapdh. n = 3 mice per group. Error bars in c and f represent s.e.m.
Extended Data Figure 8 | Formation of apoptotic bodies but not string-like apoptopodia structures is dependent on actomyosin contraction.

a, Time-lapse images monitoring apoptotic cell morphology of cells treated with or without CBX (500 μM) and in the presence of actomyosin contraction inhibitors. Top right, percentage of apoptotic cells forming string-like apoptopodia structures (387, 414, 459 and 372 apoptotic cells were analysed for Y-27632, Y-27632 + CBX, Cyto-D and Cyto-D + CBX-treated cells, respectively, from three independent experiments). b, c, Time-lapse images monitoring apoptotic cell morphology of cells stably expressing the dominant-negative PANX1 mutant (PANX DN mutant) (b) or treated with 40 μM trovafloxacin (c) in the presence of Cyto-D (5 μM). d, Inhibitors of blebbing, Y-27632, blebbistatin, or cytochalasin D (Cyto-D) reduce the formation of apoptotic bodies in Jurkat cells expressing PANX1 DN mutant (n = 3). e, Generation of apoptotic bodies by dying cells treated with Y-27632 (10 μM), blebbistatin (50 μM) and Cyto-D (5 μM). Cells were induced to undergo apoptosis in the presence or absence of CBX (500 μM) (n = 3). f, The enhanced formation of apoptotic bodies in apoptotic thymocytes from mice with PANX1 deficiency is also blunted by the ROCK inhibitor Y-27632 (10 μM) that blocks membrane blebbing (n = 3). Error bars represent s.e.m. Scale bars, 5 μm. Arrows, apoptopodia.
Extended Data Figure 9 | Inhibition of pannexin 1 during ultraviolet-induced apoptosis in LR73 fibroblasts promotes the formation of membrane protrusions and apoptotic bodies. a, ATP levels in supernatants of LR73 fibroblasts treated with 40 uM trovafloxacin with or without apoptosis induction (n = 3). b, Formation of apoptotic bodies (left) and TO-PRO-3 uptake (right) by LR73 fibroblasts treated with the indicated concentrations of trovafloxacin (n = 3). c, Generation of apoptotic bodies by LR73 fibroblasts treated with 2 mM probenecid (n = 3). d, Time-lapse images monitoring apoptotic cell morphology of LR73 fibroblasts treated with or without trovafloxacin (40 uM) or probenecid (2 mM). LR73 fibroblasts were induced to undergo apoptosis by ultraviolet treatment in all indicated experiments. Error bars in a–c represent s.e.m. Arrows, apoptopodia. Scale bars, 10 μm.
Extended Data Figure 10 | Schematic diagram depicting where pannexin 1 likely acts in limiting the fragmentation of apoptotic cells. Blocking PANX1 function (for example via trovafloxacin) leads to formation of apoptopodia, and subsequently the release of apoptotic bodies.