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Use of chemical probes to explore the toxicological potential of the K^+/Cl^- cotransporter (KCC) as a novel insecticide target to control the primary vector of dengue and Zika virus, *Aedes aegypti*

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ABSTRACT

The majority of commercialized insecticides target the insect nervous system and therefore, neural proteins are well-validated targets for insecticide development. Considering that only a few neural targets are exploited for insecticidal action and the development of insecticide resistance has reduced the efficacy of current insecticidal classes, we sought to test the toxicological potential of the potassium-chloride cotransporter (KCC). In mammals, KCC proteins have seminal roles in shaping GABAergic signaling and inhibitory neurotransmission, thus ion transport through KCC is critical for proper neurotransmission. Therefore, we hypothesized that mosquito KCC represents a putative insecticide target site and that pharmacological inhibition of KCC constructs in *Aedes aegypti* will be lethal. To test this hypothesis, we developed a robust, cell-based fluorescence assay that enables *in vitro* characterization of small-molecules against *Ae. aegypti* KCC and performed a proof-of-concept study employing well characterized mammalian KCC modulators to determine the toxicological potential of *Ae. aegypti* KCC. The selective inhibitor of mammalian KCC, termed VU0463271, was found to be a potent inhibitor *Ae. aegypti* KCC and microinjection induced lethality in a concentration-dependent manner to susceptible and pyrethroid resistant strains. Importantly, an analog of VU0463271 was shown to be > 40-fold less potent and did not induce toxicity, suggesting that the observed physiological effects and mortality are likely due to KCC inhibition. This proof-of-concept study suggests that *Ae. aegypti* KCC represents a putative target site for mosquitocide design that can mitigate the current mechanisms of insecticide resistance.

1. Introduction

Mosquitoes are vectors of numerous pathogens that cause diseases of significant relevance to global health. For instance, the yellow fever mosquito, *Aedes aegypti,* is the primary vector of the arboviruses that cause Zika, Dengue, Mayaro and Chikungunya fevers, which are four important diseases that are emerging and/or reemerging around the globe. Combined, these arboviruses are responsible for hundreds of thousands of hospitalizations and deaths per year and endemic areas are forced to shoulder substantial economic burdens with annual costs in the billions of dollars [1–3]. Effective, low-cost therapeutics and/or vaccines for treating and preventing these diseases have either not been developed or are not widely available. Similarly, biological and genetic approaches to control mosquito populations are growing in popularity [4,5], yet these options are not logistically feasible and are cost prohibitive at the present time. Therefore, reducing the burdens of mosquito-borne pathogens relies almost exclusively on the use of synthetic insecticides. The majority of insecticides used for mosquito control target the voltage-gated sodium channels (e.g. pyrethroids) or the acetylcholinesterase enzyme (e.g. organophosphates, carbamates) in the nervous system [6], which has bottlenecked mosquito populations and driven the evolution of insecticide resistance to these chemical classes [7–11]. Due to this, significant efforts have been made to identify and characterize novel physiological target sites capable of inducing lethality to adult mosquitoes while mitigating the established insecticide resistance [12–16].

Despite the nervous system being the target tissue of the majority of deployed insecticides [6], a complete understanding of the

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physiological pathways critical for proper function of the insect nervous system is still lacking. This represents a critical gap in our knowledge of the complex relationship between the dozens of functionally coupled ion channels, transporters, and enzyme systems that require tight regulation for proper neuronal function. This fundamental gap must be filled to develop a holistic understanding of insect nervous system function that will lead to the development of new insecticides. One such physiological pathway that is underexplored in insects and may represent an insecticide target site is the potassium-chloride cotransporter (KCC).

Neuronal activity patterns in the CNS are shaped by inhibitory synaptic signaling pathways, which are most often mediated through anion permeable GABA or glycine ionotropic receptors [17]. In mammals, these receptors mediate hyperpolarizing inhibition of neural activity through the opening of GABA- or glycine-gated chloride (Cl^-) channels that facilitate the inward conductance of negatively charged Cl^- ions. Importantly, these hyperpolarizing currents are only possible if the neurons have a mechanism to persistently extrude Cl^- to maintain a low intracellular chloride concentration ($[Cl^-]_i$). In mammals, the neuronal specific KCC, termed KCC2, is responsible for this persistent Cl^- extrusion and has been shown to be essential for proper neural function [18,19]. Genetic knockout of KCC2 is lethal at birth and genetic knockout of the KCC2b isoform leads to spontaneous seizures and death 2–3 weeks postnatally [20]. Similarly, pharmacological inhibition of KCC2 with a selective small-molecule inhibitor, termed VU0463271, resulted in increased $[Cl^-]_i$ and persistent CNS discharge activity [21,22]. This study validated the notion that this pathway is critical for inhibitory neurotransmission and proper neural function in mammals. In insects, immunohistochemical staining has revealed expression of KCC in the *Drosophila* brain neuropil regions including the protocerebrum and deutocerebrum [23]. Further, null mutations of the gene that is homologous to mammalian KCC2, termed *Kazachoc,* was shown to cause recessive lethal phenotypes [23]. A phenotypic characterization of partial loss-of-function *Kazachoc* mutants were shown to have an increased susceptibility to seizure-like activity that was speculated to be due to the reduced functional capacity of the $GABA_A$ receptor [23]. These data support the notion that neural KCC function is conserved amongst insects and mammals and represents a critical physiological pathway that is required for proper nervous system function.

Considering 1) the nervous system is the target tissue for the majority of deployed mosquitocides, 2) that KCC represents a critical transport pathway in the *Drosophila* and mammalian central nervous systems, and 3) *Aedes aegypti* has been shown to express KCC encoding genes [24], we hypothesized that mosquito KCC represents a putative insecticide target site and that pharmacological inhibition of KCC constructs in *Aedes aegypti* will be lethal. To test this hypothesis, we developed a robust, cell-based fluorescence assay that enables *in vitro* characterization of small-molecules and performed a proof-of-concept study employing well-characterized mammalian KCC2 modulators to determine the toxicological potential of *Aedes aegypti* KCC.

2. Methods

2.1. Compounds and compound synthesis

VU0463271 was acquired from Tocris Bioscience (Minneapolis, MN) and the VU0463271 analog 44BD was synthesized as described previously [25,26] and stored in DMSO. Molecular structures of VU0463271 and 44BD are shown in Fig. 1.

2.2. Aedes aegypti KCC (AaKCC)-overexpressing cell line generation

2.2.1. AaKCC subcloning

We generated a vector suitable for stable expression of *Aa*KCC in human embryonic kidney 293 (HEK293) cells by cloning the ORF of

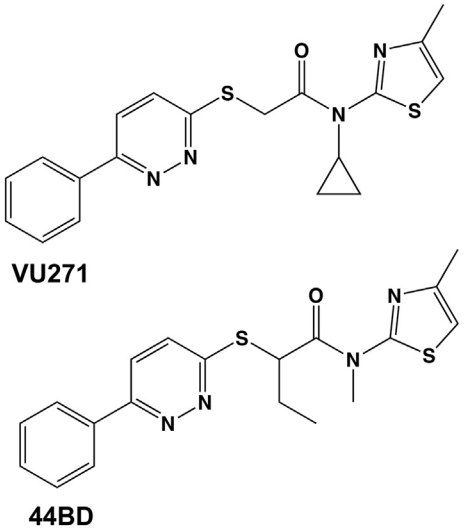

Fig. 1. Chemical structures of KCC inhibitors used in this study.

*Aa*KCC1-A1 [24] (GenBank accession number HM125960.1) into pCMV6-A-Hygro (OriGene, Rockville, MD). The *Aa*KCC ORF was synthesized in gBlocks Gene Fragments (Integrated DNA Technologies, Skokie, Illinois) with appropriate ends for Gibson Assembly [27] into pCMV6-A-Hygro. The pCMV6-A-Hygro backbone was digested with *Asi*SI and *Mlu*I (New England Biolabs, Ipswich, MA) and the *Aa*KCC fragments were cloned into the digested backbone using Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA). The assembled DNA was transformed into DH5α *E.coli* (Vanderbilt Molecular Cell Biology Resource Core). The resulting plasmid DNA (*Aa*KCC-pCMV6-A-Hygro) was purified with commercial kits and its sequence was confirmed by Sanger sequencing (GenHunter, Nashville, TN).

2.2.2. Monoclonal AaKCC cell line generation

HEK293 cells were plated in a TC-treated T75 flask at 40% confluence in α-MEM (Corning, Corning, NY) containing 10% (v/v) fetal bovine serum (ThermoFisher, Waltham, MA) and 1× Glutagro (Corning, Corning, NY) (Cell Culture Medium) and transfected with 7 μg of *Aa*KCC-pCMV6-A-Hygro DNA using Fugene6 (Promega, Madison, WI) following the manufacturer's instructions. Forty-eight hours after transfection, 250 μg/μL hygromycin (Corning, Corning, NY) was added to the medium. After two weeks of hygromycin selection, the polyclonal cells were dislodged from the flask using TrypLE Express (ThermoFisher, Waltham, MA) collected, counted, and plated in 96 well plates at ~1 cell per well in 80 μL/well Cell Culture Medium containing 250 μg/μL hygromycin. Monoclonal cell lines were assayed using Tl^+ flux [26] and known KCC-family inhibitors, bumetanide [26] and VU0463271 [25] to assess *Aa*KCC expression. The monoclonal cell line (1F4) was selected based on the magnitude of KCC inhibitor-sensitive Tl^+ flux relative to untransfected HEK293 cells. KCC expression in the 1F4 monoclonal cell line was confirmed by Western blotting (data not shown). The 1F4 monoclonal cell line was used for all experiments in this manuscript.

2.3. Cell culture of HEK293 cell lines expressing KCC

Wild-type and monoclonal *Aa*KCC-expressing HEK293 cells were grown to 80%–90% confluence in TC-treated T75 flasks containing Cell Culture Medium. *Aa*KCC-expressing cells were grown in the presence of 250 μg/μL hygromycin. Cells were passaged when they reached approximately 80%–90% confluence at a ratio of 1:10, for a maximum of 20 passages.

2.4. Thallium (Tl^+)-flux assays

Tl^+ flux assays were performed based on previously described methods [26]. The day before the experiment, cells were plated in black-walled, clear-bottom, 384 well BD PureCoat amine-coated plates (BD, Bedford, MA) at a concentration of 20,000 cells/well. On the day of the experiment, the HEK293 Cell Culture Medium was removed and replaced with 20 µL/well of Hank's Buffered Salt Solution (Thermo-Fisher, Waltham, MA) plus 20 mM HEPES-NaOH, pH 7.3 (Assay Buffer) containing 1.25 ng/µL Thallos-AM plus 0.02% final Pluronic F-127 (Millipore Sigma, St. Louis, MO) (Dye Loading Solution). Cells were incubated in Dye Loading Solution for 1 h at room temperature in the dark. After incubation, the Dye Loading Solution was replaced with 20 µL/well Assay Buffer. Compound serial dilutions were made in dimethyl sulfoxide (DMSO) (Millipore Sigma, St. Louis, MO) and then transferred to Assay Buffer at 2-fold above the final desired concentration. Tl^+ flux was measured using the Panoptic (Wavefront Biosciences, Franklin, TN) kinetic imaging plate reader (1 Hz, excitation 480/40, emission 538/40). Following collection of 10 frames of baseline images, imaging continued with the addition of 20 µL/well of 2× compound solution. After four minutes of incubation with continuous imaging, 10 µL/well of 125 mM sodium bicarbonate, 12 mM thallium sulfate, 1 mM magnesium sulfate, 1.8 mM calcium sulfate, 5 mM glucose, and 10 HEPES-NaOH (pH 7.3) (Millipore Sigma, St. Louis, MO) were added to the cells and imaging was continued for two additional minutes.

2.5. Mosquito colony

An established colony of *Ae. aegypti* mosquitoes, Rockefeller strain, was reared and maintained in an environmental chamber at 28 °C and 70% relative humidity with a 12:12 dark and light cycle at Louisiana State University (Baton Rouge, LA, USA). Mosquitoes were originally provided by Dr. Todd Walker at the East Baton Rouge Mosquito Control and Abatement.

Eggs from a pyrethroid-resistant strain of *Ae. aegypti*, Puerto Rico strain (PR, NR-48830), were obtained from BEI Resources, NIAID, NIH and reared to adults. Third-instar larvae of the resistant strain of *Ae. aegypti* were exposed to permethrin (0.1 mg/mL) (Millipore Sigma, St. Louis, MO) every third generation to maintain the resistance trait (personal communication, Mr. Paul Howell, Centers for Disease Control and Prevention, Atlanta, GA). Adult mosquitoes of all strains were fed a 10% sucrose solution *ad libitum* and held under the same conditions as the Rockefeller strain. All experiments were carried out on adult females at 3–5 days post-eclosion.

2.6. Adult mosquito toxicology experiments

For topical toxicity assays, the method of Pridgeon et al. [28] was used with slight modifications. Briefly, mosquitoes were chilled on ice for 3 min, during which 200 nL of chemical (dissolved in 95% ethanol) was applied onto the pronotum using a handheld microapplicator (Hamilton Co., Reno, NV, USA). For each chemical, 5 doses with 10 mosquitoes per dose was repeated 2–3 times using different batches of mosquitoes.

For injection toxicity assays, chemicals were dissolved in DMSO and adult female mosquitoes were anesthetized on ice to enable manipulations. The mosquito was impaled through the metapleuron using a pulled-glass capillary attached to a Nanoliter2010 with Micro 2 T injection system (World Precision Instruments, Sarasota, FL). Each mosquito received a single injection of 69 nL of solution containing the chemical or DMSO (control). The injection solution consisted of a calcium-free phosphate-buffered saline (Thermo Fisher, Waltham, MA). DMSO concentrations did not exceed 1% in the highest dose administered. After injection, mosquitoes were placed into 20 × 20 × 20 cm screen cages within a rearing chamber, and allowed free access to 10% sucrose. Mortality was assessed at 24 h post injection. ID_{50} determinations were based on testing at least nine test compound doses that consisted of three replicates of 10 adults per concentration, which was repeated on three separate cohorts. Each concentration consisted of at least 90 individuals.

2.7. Larval mosquito toxicology experiments

We used two distinct larval toxicity bioassays to assess pharmacokinetics of KCC modulators and toxicity. First, we employed a 24 h bioassay on intact larvae to determine if compounds could penetrate the integument to cause toxicity. Methods followed those described previously [29,30]. Ten fourth instar larvae per treatment condition were placed in dishes containing a larval saline that consisted of: 154 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $NaHCO_3$, (pH 6.9) plus DMSO or with test compound dissolved in DMSO. Larvae were observed after 1 h for any immediate toxic effects. Larvae were evaluated after 24 h of exposure by providing a mechanical stimulus to determine paralysis, which was determined by a body wall contraction or any movement. A lack of movement or contractility to the mechanical stimulus was scored as dead. LC_{50} determinations were based on testing at least six test compound concentrations that consisted of three replicates of 10 larvae per concentration, which was repeated on three separate cohorts. Mortality data for each concentration were pooled for each cohort and three LC_{50} values were determined and the average was determined between the 3 replicates. The LC_{50} was determined using GraphPad Prism (GraphPad Software, San Diego, CA). Each concentration consisted of at least 90 individuals.

For the second larval toxicity assessment we employed a headless larva bioassay to circumvent the problem of low cuticular penetration and provide a better estimate of the intrinsic toxicity of test compounds [31]. These methods were performed as previously described in Islam and Bloomquist [31]. Decapitation of 4th instar larvae was performed with forceps. The larvae were then placed into a test compound/vehicle/saline solution and observed for toxic effects every hour for a period of 5 h. Headless larvae show a strong bilateral contractile motion when probed and therefore, mortality was defined by no movement or a sluggish, unilateral contractility. Mortality was assessed at 1-, 3-, and 5-h after initial removal of the head. Vehicle controls were performed for each test compound concentration and data were discarded if control mortality exceeded 10%.

2.8. Diuresis experiments in Aedes aegypti

The excretory capacity of adult female *Ae. aegypti* (LVP strain) was measured as described previously [12,13]. Groups of 10 mosquitoes were injected with 200 nL of VU0463271 (1 µg/mg mosquito), 44BD (100 µg/mg mosquito), or 1% DMSO 2 h before injecting the hemolymph of each mosquito with 69 nL of a potassium-enriched, phosphate-buffered saline (K^+-PBS). The K^+-PBS consisted of the following (in mM): 92.2 NaCl, 47.5 KCl, 10 Na_2PO_4, and 2 KH_2PO_4 (pH 7.5). Each treatment group of mosquitoes was transferred into a separate graduated, packed-cell volume tube (MidSci, St. Louis, MO) and incubated for 1 h at 28 °C. The volume excreted by the mosquitoes was measured visually via the graduated column at the bottom of the tube. For each treatment, 40 female mosquitoes were exposed to the chemical. These treatments were repeated on four separate broods. The mean volume excreted for each individual cohort was averaged and plotted. All mosquitoes were confirmed to be alive at the end of 1 h and any dead mosquitoes were discarded. The mean ($n = 4$) volumes excreted by vehicle-, VU0463271-, furosemide (Millipore Sigma, St. Louis, MO)-, and 44BD-treated mosquitoes were analyzed using a one-way ANOVA with a Newman-Keuls *post hoc* analysis using GraphPad Prism.

2.9. Statistical analyses

2.9.1. Tl^+-flux assay

Fluorescence values were normalized on a well-to-well basis by dividing the fluorescence values at each time point by the average of the baseline fluorescence values (F/F_0) for a given well. The slope of the fluorescence increase between 1 and 86 s after Tl^+ addition was used as a measure of Tl^+ flux. The slopes of the change in fluorescence in the absence of inhibitor were designated as 100% KCC activity and slopes obtained in the presence of a maximally effective concentration of 40 μM were designated as 0% KCC activity. To compare the effect of DMSO on *Aa*KCC-mediated Tl + flux, a one-way ANOVA was performed with a Tukey's multiple comparison test. The potency of test compounds was determined by fitting slopes obtained at compound concentrations varying over a range of 40 μM to 0.03 nM using a four parameter logistic equation with GraphPad Prism. For a given experiment, all conditions were tested in six wells per plate. Values reported are averages of those obtained from three independent experiments +/− standard error of the mean (SEM).

2.9.2. Mosquito toxicology

Mortality of adults and intact larvae were recorded 24 h post-treatment whereas, headless larvae toxicity was determined at 1-,3-,5- h post exposure. ID_{50}/LC_{50} were calculated using a nonlinear regression curve fitting with GraphPad Prism (GraphPad Software, San Diego, CA). Three ID_{50}/LC_{50} values were obtained from separate cohorts, and the mean ID_{50} value was used for statistical analysis. For all toxicity assays, control mortality was < 10% and was corrected for using Abbot's formula [32].

3. Results

3.1. Development of an AaKCC expressing cell line for in vitro testing

In order to facilitate *in vitro* characterization of the effects of test compounds on *Aa*KCC, we constructed a HEK293 cell line that stably expresses *Aa*KCC. Previously we developed a Tl^+ flux assay to measure the activity of human KCC (hKCC) [26]. The Tl^+ flux assay reports the KCC-facilitated flux of the K^+ congener, Tl^+, across the plasma membrane using the intracellular Tl^+-sensitive fluorescent dye Thallos (WaveFront Biosciences). Fig. 2A shows a representative fluorescence trace recorded from an individual well of a 384-well plate containing *Aa*KCC-expressing HEK293 cells in the absence of inhibitors. Addition of Tl^+ evoked a rapid fluorescence increase in untreated *Aa*KCC-expressing cells that was inhibitable with small-molecule modulators (Fig. 2B). For instance, the addition of bumetanide, a non-selective inhibitor of mammalian KCC, decreased Tl^+ flux in *Aa*KCC cells to the level of Tl^+ flux observed in wild-type HEK293 cells (Fig. 2B). These data demonstrate that our Tl^+ flux assay and *Aa*KCC-expressing cell line are useful for measuring the effects of pharmacological modulators on *Aa*KCC activity.

The assay was validated for *in vitro* screening by meeting a series of performance benchmarks. First, the assay was tested for its tolerance to the small-molecule vehicle DMSO at concentrations up to 10% v/v. As shown in Fig. 2C, the Tl^+-flux mediated by *Aa*KCC is unaffected by DMSO concentrations up to 0.625% DMSO v/v as compared to the 0% DMSO control (one-way ANOVA, $P > 0.05$). A 20 ± 15% and 80 ± 9% inhibition of KCC-mediated fluorescence was observed at DMSO concentrations of 1.25% and 2.5% DMSO, which were statistically significant reductions ($P < 0.05$). Interestingly, 5% DMSO yielded greater Tl + −flux when compared to 2.5% DMSO with a 49 ± 10% inhibition of Tl^+-flux (Fig. 2C). KCC-mediated fluorescence was reduced at a concentration of 5% DMSO. The data indicate that the developed cell line and assay are robust and capable of generating reproducible data for *in vitro* screening of *Aa*KCC modulators.

3.2. VU0463271 is a potent inhibitor of AaKCC

The non-selective nature and low potency of bumetanide make it unsuitable for exploring the toxicological potential of KCC inhibition. Therefore, we sought to determine whether a recently describe highly potent, highly selective hKCC inhibitor, VU0463271 [25,26], is capable of inhibiting *Aa*KCC. Using the aforementioned Tl^+ flux assays, VU0463271 was shown to inhibit *Aa*KCC in a concentration-dependent manner. Curve fits to varying concentrations of VU0463271 yielded an IC_{50} of 1.3 μM (95% CI: 0.9–1.8 μM) and a Hill coefficient value of 1.3 (Fig. 2D). These data demonstrate that VU0463271 is in fact an inhibitor of *Aa*KCC. In contrast, VU0463271 did not show an appreciable effect on the Tl^+ flux in wild-type HEK293 cells.

In an effort to identify a closely related inactive analog of VU0463271 for use as a negative control in our toxicology studies, we chose a compound, 44BD, which was previously shown to possess very low inhibitory activity at hKCC [25]. When we evaluated 44BD using our *Aa*KCC Tl^+ flux assay we observed a dramatic loss in potency when compared to VU0463271 with only a 25 ± 10% inhibition at 40 μM, which was the highest concentration tested (Fig. 2D). These data closely match what was observed with hKCC and verify that 44BD is a suitable "inactive" analog to act as a negative control in our *in vivo* studies exploring the physiological effects of VU0463271 on mosquitoes.

3.3. Toxicity of VU0463271 to Aedes aegypti

The toxicity of VU0463271 and 44BD was first assessed through topical bioassays, yet < 30% mortality was observed after topical exposure at solubility limits. Therefore, we bypassed the cuticular barrier by microinjection of VU0463271 and observed a concentration dependent effect to mortality with an ID_{50} of 56 ng/mg of mosquito (95% CI: 39–107 ng; Hillslope: 0.79; r^2: 0.93) (Fig. 3A). 44BD yielded significantly lower mortality when compared to VU0463271 with approximately 20% mortality at 500 ng, which was the highest dose studied due to solubility limitations (Fig. 3A). In addition to the Rockefeller strain (susceptible), we also employed the Puerto Rico (PR) strain of *Aedes aegypti* which has been shown to possess target-site (*kdr*) resistance (BEI resources), which contrasts from another Puerto Rican strain that possesses elevated mRNA levels encoding CYP450 enzymes [9]. Importantly, the ID_{50} of PR strain, when exposed to VU0463271, was 1.3-fold reduced when compared to the Rockefeller strain (Fig. 3A), which was not a statistically significant difference in toxicity (Fig. 3A). These data are consistent with the dramatically lower potency of 44BD compared to VU0463271 observed in our *in vitro* assays.

Larval toxicity assays provided similar results when compared to adult toxicity in that no mortality was observed in intact larvae at concentrations up to 200 parts-per-million (ppm) (data not shown). To bypass the cuticular and blood brain barriers, we performed the headless larval assay described in Islam and Bloomquist [31]. Similar to the toxicity observed in adult mosquitoes, VU0463271 showed concentration-dependent lethality with an LC_{50} value of 47 ppm (95% CI: 39–56 ppm; Hillslope: 1.1; r^2: 0.94) at 1-h post exposure (Fig. 3B). We observed a 3.3-fold increase in toxicity at hour 3 (LC_{50} 14.9 ppm, 95% CI: 12–18; Hillslope: 1.0; r^2: 0.95) and a 23.5-fold increase in toxicity at hour 5 (LC_{50} 2.0 ppm, 95% CI: 1.1–3.8; Hillslope: 0.97; r^2: 0.94) when compared to hour 1 (Fig. 3B). Importantly, 44BD was found to be non-toxic to intact larvae and was significantly less toxic to headless larvae when compared to VU0463271 (Fig. 3C). We were unable to determine an LC_{50} due to lower than 50% lethality observed at the highest concentration tested. At 1- and 3-h post exposure, we observed a mean mortality of 18 ± 48% and 42 ± 19% at 300 ppm, respectively. Three-hundred ppm was the highest concentration tested due to solubility limits (Fig. 3C). At 5-h post 44BD exposure, we observed 58 ± 16% mortality at 300 ppm and estimated the LC_{50} to be approximately 230 ppm, which is a 115-fold decrease in toxicity when compared to the LC_{50} of VU0463271 at hour 5 (Fig. 3C). The toxicity

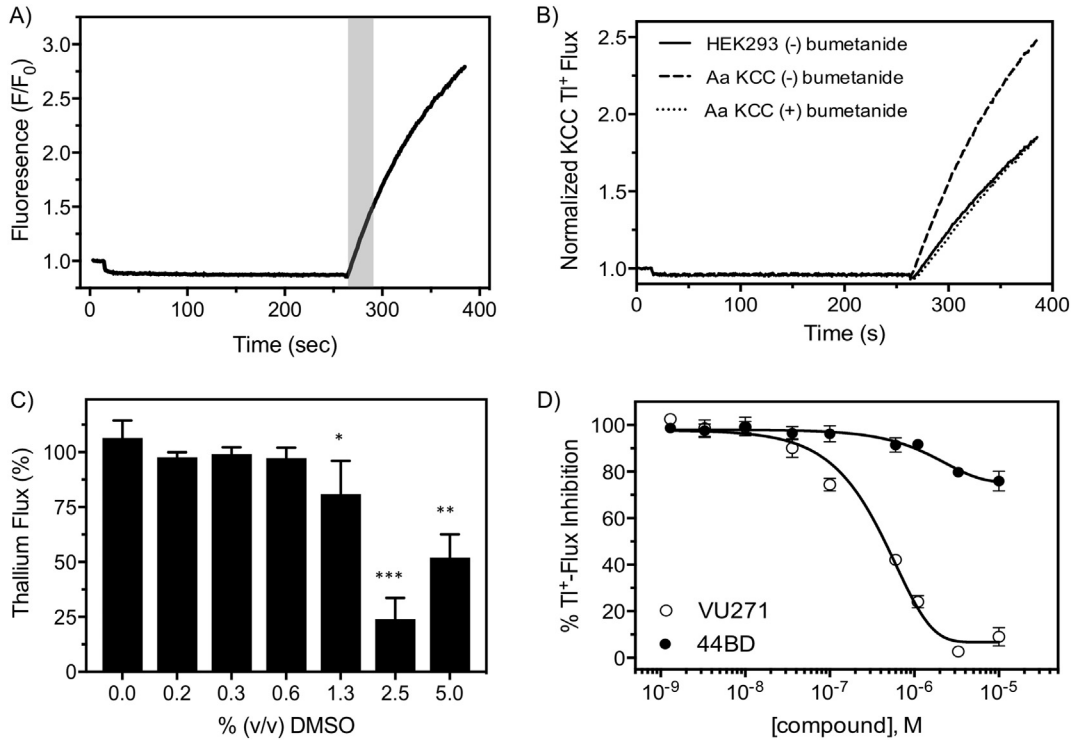

Fig. 2. Development of an *Aedes aegypti* KCC expressing cell line for *in vitro* testing of pharmacophores. (A) Representative Tl^+-induced fluorescence of a single well of cells containing HEK293-AeKCC-expresing cells. The shaded box indicates the cell exposure to Tl^+. (B) Representative Tl^+-induced changes in fluorescence in the absence and presence of bumetanide. Treatments were: HEK293 cells exposed to bumetanide but not expressing *Ae*KCC (solid line), HEK293 cells expressing *Ae*KCC not exposed to bumetanide (dashed line), and HEK293 cells expressing *Ae*KCC exposed to bumetanide (dotted line). (C) DMSO concentrations up to 5% v/v DMSO. Data are means (n = 6) and were compared to 0% DMSO *via* a one-way ANOVA. Statistical significance denoted by an asterisk where * represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$. (D) Concentration-response curve of VU0463271 (open circles) and 44BD (closed circles) derived from Tl^+ flux assays. Data points are mean (n = 3) independent experiments performed in triplicate.

profiled observed with VU0463271 and 44BD in the mosquito larvae assay corresponds to the potency and toxicity profiles observed for the *in vitro* and adult mosquito toxicity assays, respectively.

3.4. Signs of intoxication with VU0463271

After treatment with lethal doses of VU0463271, *Aedes aegypti* mosquitoes were found to display hyperexcitatory tendencies. In adults, 20 min after treatment, the mosquitoes were observed to display an increased wing beat frequency, erratic and uncoordinated movements, reduced climbing behavior, and increased leg contractions. Directed flight behavior did not occur. After approximately 10 s of hyperexcitation, the mosquitoes resumed lethargic behavior. Control mosquitoes had normal posture (legs not splayed away from the midline), would rest on the sides of the holding chamber *versus* the bottom, and upon agitation, the mosquitoes would immediately fly from their resting posture to a different location on the container and come to rest. Interestingly, slight excitatory tendencies were observed in about 20% of the mosquitoes injected with 44BD but were not to the same level of intensity when compared to mosquitoes treated with VU0463271. This modest efficacy is consistent with dramatically lower potency but not complete inactivity of 44BD against *Aa*KCC (Fig. 2D).

3.5. Effects of VU0463271 on the excretory capacity of adult female Aedes aegypti

Since KCC1-A, which is related to the mammalian KCC isoforms important for salt homeostasis in the kidney, has been shown to be a critical pathway that is essential for proper function of mosquito Malpighian tubules and fluid secretion from the isolated tubules [24], we evaluated the effects of VU0463271 on the excretory capacity of adult *Aedes aegypti*.Fig. 4 shows the volume of urine excreted by mosquitoes 1 h after injection with vehicle, VU0463271, or 44BD. Compared to injection of the vehicle, VU0463271 significantly ($P < 0.01$) reduces the amount of excreted urine by 55%, whereas urine secretion in 44BD treated mosquitoes was not significantly different from animals treated with vehicle alone.

4. Discussion

The SLC12 gene family encodes cation-coupled Cl^- cotransporters, such as KCC2, and has received relatively little attention within the field of insecticide science despite the increased interest in identification of novel target sites for insecticide design. To date, pharmacological studies have revealed that inhibition of KCC1 reduces the transepithelial fluid secretion by the Malpighian tubules of *Rhodnius prolixus* [33,34], *Drosophila melanogaster* [35], and *Aedes aegypti* [24], suggesting KCC may represent a putative insecticide target site by altering blood meal digestion and osmoregulation to induce mortality, similar to the previous work on mosquito inward rectifier potassium channels [12]. Additionally, genetic depletion of the *Kazachoc* gene in *Drosophila*, which is analogous to the neural KCC2 transporter in mammals, leads to neurological excitability by preventing inhibition of neurotransmission [23]. A similar linkage between neural KCC and regulation of inhibitory neurotransmission was also observed with *Caenorhabditis elegans* [36]. Although limited in scope, the current body of work for invertebrate KCCs raises the intriguing possibility that pharmacological inhibition of insect KCC proteins will lead to altered neurotransmission that results in mortality. Yet, to date there have been no published studies describing the potential of neural KCC to represent an insecticide target site for control of arthropod pests. Indeed, the results of the present study provide compelling data suggesting that KCC is a

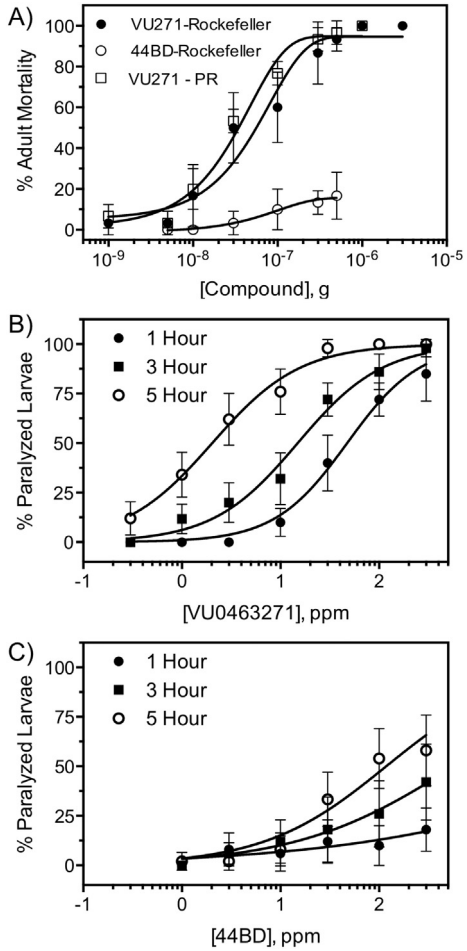

Fig. 3. Toxicological characterization of VU0463271 and 44BD in *Aedes aegypti*. (A) Toxicity of VU0463271 and 44BD to susceptible (Rockefeller; closed and open circles) and pyrethroid resistant (Puerto Rico; open square) strains of adult *Aedes aegypti* 24 h post-injection. Data points represent an n = 90 individual female mosquitoes. (B) Toxicity of VU0463271 to headless larvae at 1 h (closed circle), 3 h (closed square), and 5 h (open circle) post-exposure. Data points represent mean mortality where n = 30 individuals. (C) Toxicity of 44BD to headless larvae at 1 h (closed circle), 3 h (closed square), and 5 h (open circle) post-exposure. Data points represent mean mortality where n = 30 individuals.

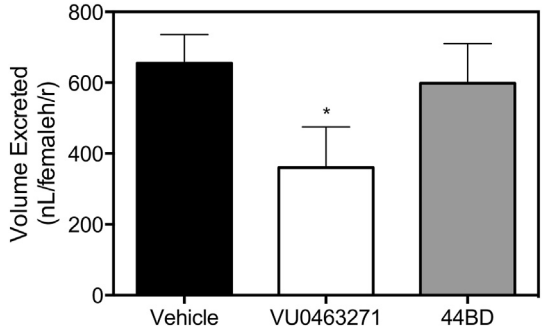

Fig. 4. Effects of VU0463271 and 44BD to the excretory capacity of adult female *Aedes aegypti*. Amount of urine excreted by mosquitoes 1 h after injection with 69 nL of the vehicle control or VU0463271/44BD solutions in K^+-PBS. Bars represent mean (n = 40) where error bars represent SEM. Statistical significance was denoted by the asterisk where * represents $P < 0.05$.

critical physiological pathway in the *Aedes aegypti* nervous system and represents a putative target site for insecticide design.

Prior to 2009, KCC pharmacology was limited, with the loop-diuretics furosemide and bumetanide representing the two best pharmacological probes of KCC function. However, these molecules have low KCC activity, are poorly selective for KCC, and have poor pharmacokinetics that limit *in vivo* studies in insects. To remedy this issue, Delpire and colleagues performed a high-throughput screen against mammalian KCC2 and identified the small-molecule termed ML077, which was 1000-fold more potent than bumetanide [26]. Modifications to the ML077 structure resulted in the development of a VU0463271 that is 9-fold greater in potency at human KCC2 and is selective against the related transporter, Na^+-K^+-$2Cl^-$ cotransporter 1 [25], which is an advantageous property for *in vivo* studies of mammals and insects. The identification of selective and potent small-molecules designed to target mammalian KCC2 has enabled researchers to begin to characterize the physiological role of these transporters in various tissue systems and in non-human organisms.

In this study, we first sought to determine whether the potent and selective mammalian KCC2 inhibitor (VU0463271) is able to inhibit *Aa*KCC. We found that VU0463271 did in fact inhibit *Aedes aegypti* KCC function, albeit with 16-fold less potency when compared to humans [25]. The difference in potency between species is not surprising, given that hKCC2 and *Aa*KCC only share 52% amino acid sequence identity. The fact that VU0463271 displayed strong preference for hKCC over *Aa*KCC suggests it is possible to develop an insect KCC inhibitor that displays high selectivity for *Aa*KCC over hKCC by exploiting the molecular differences of the two proteins in a similar manner to the pyrethroid class of insecticides. For instance, permethrin is > 100-fold more potent at the insect voltage-gated sodium channel *para* than its mammalian homolog, which share 67% amino acid sequence identity [37]. We also found that 44BD, which is structurally related to VU0463271, possessed poor inhibitory potency against *Aa*KCC. This matches that activity profile observed for mammalian KCC2 [25] and thus, provides a valuable negative control for subsequent *in vivo* studies.

To begin determining if KCC represents a physiological pathway that is important for proper nerve function in insects that, when poisoned, leads to insect mortality, we exposed larval and adult mosquitoes to various concentrations/doses of VU0463271. Our data show that VU0463271 does indeed produce lethality in both larvae and adult mosquitoes while 44BD showed > 100-fold less activity. Since the justification for identifying novel insecticide targets is to mitigate the established resistance to deployed insecticides, we aimed to determine if lethality induced by VU0463271 was different between a susceptible and resistant strain of *Aedes aegypti*. *A priori*, one would not expect a mosquito strain with only target-site resistance in Na^+ channels, such as the PR strain of *Ae. aegypti* used in this study, to exhibit resistance to a small molecule inhibitor of KCC channels. As such, VU0463271 showed near identical toxicity against the Rockefeller and PR strains of *Ae. aegypti,* suggesting KCC may represent a target site capable of mitigating the established target site resistance mechanisms.

KCC2 is expressed in the nervous system of mammals [38,39] and *Drosophila* [23] and therefore, we anticipate that the mechanism of toxicity is due to inhibition of neurotransmission of adult and larval mosquitoes. VU0463271 is a highly potent and selective inhibitor of human KCC2 [25] and we have shown high nanomolar to low micromolar affinity of VU0463271 to the mosquito KCC2 (Fig. 2D). Further, signs of intoxication occurred within minutes of VU0463271 exposure and toxicity was acute, which is reminiscent of neural poisoning. However, Piermarini and colleagues have described a critical role of KCC in the function of *Aedes aegypti* Malpighian tubules [24], suggesting that it is possible to induce lethality through reduced osmoregulatory capabilities. In support of this hypothesis, we observed a reduced diuretic capacity *in vivo* after exposure to a sub-lethal dose of VU0463271, suggesting that it is at least possible that VU0463271-induced toxicity is due, at least in part, to disruption of excretory

functions mediated by Malpighian tubules.

Our study provides the first proof-of-concept that suggests *Aa*KCC represents a putative insecticide target site in mosquitoes that is capable of inducing lethality to insecticide susceptible and resistant strains of *Aedes aegypti*. Although this study employed mammalian KCC2 modulators to provide a proof-of-concept that pharmacological inhibition of insect KCC is capable of inducing lethality, the lack of potent and selective pharmacological agents targeting insect KCC is a severe limitation for further development of cation-chloride cotransporters as insecticide targets. Our data verify that VU0463271 is a viable tool compound for dissecting the physiological role of KCC in insect neural systems, yet it is incapable of being repurposed as an insecticidal candidate due to relatively low potency (~1 μM) against mosquito KCC, greater activity against human KCC2 when compared to mosquitos (Fig. 2, [25]), and poor physiochemical properties that prevents cuticular penetration (Fig. 3). The fact that VU0463271 shows over 10-fold less activity at *Aa*KCC compared to hKCC offers hope that an effort to discover and develop *Aa*KCC inhibitors could yield inhibitors with a strong preference for *Aa*KCC compared to hKCC. Our previous success using a Tl^+ flux-based high-throughput screening approach to discover and develop the most potent and effective known inhibitors of mammalian KCC [25,26] demonstrates the viability of this approach to rapidly discover compounds capable of inhibiting KCC. Our present demonstration of a robust, effective, high-throughput, Tl^+ flux-based screening methodology for measuring the activity of *Aa*KCC will enable the search for KCC inhibitors which possess the potency, selectivity and physiochemical properties to allow exploration of the true potential of KCC as a novel target for the development of insecticides.

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Author contributions

Conceived, designed, and performed experiments: DRS, ZL, RC, FJP, CDW, CWR, CWL, CDW. Analyzed the data: DRS, ZL, RC, FJP, CDW. Participated in writing of the manuscript: DRS, FJP, CDW.

Conflict of interest

DRS, ZL, RC, FJP, CWR, CWL declare no conflict of interest. CDW is an owner of WaveFront Biosciences and receives royalties from the sales of Thallos-AM via a license from Vanderbilt University.

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