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A Cell-Based Screening Platform Identifies Novel Mosquitocidal Toxins

Melissa A. O'Neal¹, Bruce A. Posner², Craig J. Coates³, and John M. Abrams¹

¹University of Texas Southwestern Medical Center, Department of Cell Biology, Dallas, TX, USA

²University of Texas Southwestern Medical Center, Department of Biochemistry, Dallas, TX, USA

³Texas A&M, Department of Entomology, College Station, TX, USA

Abstract

Pesticides currently in widespread use often lack species specificity and also become less effective as resistance emerges. Consequently, there is a pressing need to develop novel agents that are narrowly targeted and safe to humans. A cell-based screening platform was designed to discover compounds that are lethal to mosquito (*Anopheles* and *Aedes*) cells but show little or no activity against other insect (*Drosophila*) or human cell lines. Mosquito-specific, aqueous-stable cytotoxins were recovered at rare frequencies. Three of these were profiled for structure-activity relationships and also assessed in whole-animal toxicity assays. In at least one test case, species-specific cytotoxicity seen in culture effectively translated to the whole-animal level, with potent toxicity against *Anopheles* yet none against *Drosophila*. Therefore, this initiative has the potential to advance novel mosquitocidal agents and, in a broader sense, could establish a versatile platform for developing customized pesticides that selectively target other disease vectors as well.

Keywords

agricultural; animal health; cell-based assays; high-content screening; phenotypic drug discovery

Introduction

As vectors of both parasitic and viral diseases, mosquito populations are a global concern. There is a broad consensus that new agents for controlling this insect vector will be needed to ultimately eradicate diseases such as malaria, helminthiasis, hemorrhagic fevers, and encephalitis.^{1–4} *Anopheles gambiae*, for example, is the predominant vector for malaria in Africa and contributes to the death of approximately one million people each year.⁵ The use of pesticides on long-lasting impregnated nets and additional mosquitocidal strategies can successfully reduce mortality rates.⁵ However, the efficacy of these strategies is limited because resistance emerges quickly,^{2,6} and this trait often confers cross-protection against multiple pesticides.^{4,7} Many pesticides also exhibit broad-spectrum toxicities. Therefore, major challenges that shape the development of new pesticides are ensuring human safety,

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Corresponding Author: John M. Abrams, PhD, Department of Cell Biology, University of Texas Southwestern Medical Center at Dallas, 6000 Harry Hines Blvd., Dallas, TX 75390-9039, USA john.abrams@utsouthwestern.edu.

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limiting off-target effects in the ecosystem, and mitigating resistance. To confront these challenges, a high-throughput cell-based screening platform was developed. The underlying rationale for approach is straightforward: Compounds lethal to cultured mosquito cells but harmless to other Dipteran or human cells would be promising leads for the development of new mosquitocidal agents that are narrowly targeted and environmentally safe. Using this strategy, mosquito-specific, water-stable cytotoxins were found at rare frequencies (0.04%), and in at least one test case, targeted activity seen in culture predicted targeted toxicity in whole-animal studies.

Previously, Pridgeon et al.⁸ reported an efficient method for whole-animal screening of mosquito larva. However, relative to cell-based screening, throughput was limited, and extensive amounts of test compounds were needed. Furthermore, the platform did not distinguish nonselective from selective toxins. The proof-of-concept study outlined here was designed to confront two pivotal assumptions. First, we tested whether we could effectively use a cell-based strategy to capture species-specific cytotoxins. Second, we tested whether targeted cytotoxins found and characterized in culture would translate to whole-animal toxicity.

Materials and Methods

Cell Lines

The mosquito line 4A3A (*Anopheles gambiae*) was kindly provided by Mario Soberon.⁹ The mosquito lines Sua 4.0 (MRA-921, *Anopheles gambiae*) and MSQ43 (MRA-858, *Anopheles stephensi*) were obtained from ATCC through the Malaria Research & Reference Reagent Resource Center (MR4), now Biodefense and Emerging Infections Research Resources Repository (BEI Resources). The two *Drosophila* lines Kc and S2R⁺ as well as the mosquito lines 4A3A and Sua 4.0 were cultured in Schneider's media with 10% fetal bovine serum (FBS), 50 U ml⁻¹ penicillin, and 50 µg ml⁻¹ streptomycin at 25 °C with no CO₂. The mosquito line Aag2 (*Aedes aegypti*) was kindly provided by Ann Fallon.¹⁰ The mosquito lines MSQ43 and Aag2 were cultured in MEM media supplemented with 1 g glucose, 2.2 g NaHCO₃, 10 mL L-glutamine 200 mM, 10 mL MEM vitamin solution 100×, 20 mL MEM nonessential amino acids, 5% FBS, 50 U ml⁻¹ penicillin, and 50 µg ml⁻¹ streptomycin at 29 °C with 5% CO₂. The human line HCT116 was provided by Bert Vogelstein¹¹ and was cultured in McCoy's media with 10% FBS, 50 U ml⁻¹ penicillin, and 50 µg ml⁻¹ streptomycin at 37 °C with 5% CO₂. The hepatic line TPH-1 was provided by Ranjit Ray.¹² The human line T98G as well as TPH-1 were cultured in DMEM supplemented with 10% FBS, 50 U ml⁻¹ penicillin, and 50 µg ml⁻¹ streptomycin at 37 °C with 5% CO₂.

Cell-Based Screening Platform

After a series of optimization trials, 4A3A cells were plated into 384-well plates at a density of 5000 cells/well and allowed to adhere for 4 h at 25 °C prior to automated delivery of the UT Southwestern chemical library compounds, all of which were at a final concentration of 5 µM (1% DMSO) in the assay. All assay plates were incubated at 25 °C for ~96 h. At the end of the incubation period, cell survival was measured using the CellTiter Glo system following the manufacturer's protocol (Promega, Madison, WI). All assay plates included reference wells with either vehicle alone (columns 2 and 23) or a lethal dose of blasticidin (500 ng mL⁻¹ in column 1). These controls enabled independent assessments of the dynamic range and assay quality of each plate as assessed using the Z' value.¹³ Plates with Z' values <0.45 were classified as failed and repeated. From this primary screen, 176 compounds inducing potent mosquito cell lethality (Z scores -3) were identified as candidates for retesting. In the second stage of the pilot study, all 176 candidates were retested in triplicate

at a final compound concentration of 5 μM (1% DMSO) against the same *Anopheles* 4A3A cell line. In parallel, these compounds were also counterscreened in triplicate against two different *Drosophila* cell lines (S2R⁺ and Kc). The three cell lines S2R⁺, Kc, and 4A3A were all plated at the same density and assessed as in the original screen. All data were analyzed and quality controlled using the Screener software suite (version 10, GeneData, Inc., Basel, Switzerland).

Compound Acquisition and Assessment Strategies

Compounds were purchased anew from the commercial entities ChemBridge and ChemDivision (listed in Supplementary Appendix Table S1). The newly purchased compounds were then confirmed against the mosquito line 4A3A as well as the two originally tested *Drosophila* lines per the screening platform described. Pipeline Pilot (version 8.5, Accelrys, San Diego, CA) was used to perform structure-based clustering for the generation of the SW120412, SW137658, and SW0497553 family groups.

Stability Assay

Compounds are routinely stored as a powder, in 100% DMSO at 10 mM or 100% DMSO at 20 mM, all at -20°C . Aliquots of the 10 mM freezer stocks were put into non-light-protecting Eppendorf tubes. In addition, these aliquots were used to make 1:50 dilutions in H₂O (200 μM) in the same type of tube. These aliquots were stored at ambient temperature for a month prior to retesting.

IC₅₀ Assay

Drosophila and mosquito cell lines were plated into 384-well plates at a density of 5000 cells/well and allowed to adhere for 4 h prior to compound addition. Mammalian cell lines were plated into 384-well plates at a density of 2000 cells/well and allowed to adhere for 24 h prior to compound addition. The Echo 555 Liquid Handler by Labcyte (Sunnyvale, CA) was used for precise automated delivery of compounds. Each compound was diluted by half-log intervals in triplicate from a top dose of 50 μM (SW1376580) or 500 μM (SW120412 and SW049753). Curve fitting was performed using the Condoseo module of the Genedata Screener (10.0.2) software suite (Genedata AG, Basel, Switzerland). The nonlinear curve-fitting algorithm in this module uses the four-parameter Hill equation. The parameters in this equation are defined as follows: the activity level at zero concentration of test compound; Sinf, the activity level at infinite concentration of compound; X, the concentration of the test compound in logarithmic units (varied in the experiment); IC₅₀, the concentration of activity at which the activity is 50% of the maximum level; and the Hill coefficient (n), a measure of the slope at the IC₅₀. The program provided a goodness of fit as determined by R^2 values for each curve. In cases in which there was no dependence of activity on the dose of the test compound, a lower bound of the IC₅₀ was set to the top dose in the experiment (e.g., >50 mM).

Whole-Animal Assay: *Anopheles* and *Aedes*

Mosquito eggs were placed in water to hatch and were maintained at 25 $^{\circ}\text{C}$ in a humidified room. After 24 h, the first instar larvae were counted and transferred into individual trays containing 500 mL water with 0.5% DMSO and the test compound at the required final concentration. Ground Tetramin tropical fish food was added each day in small amounts to ensure most of the food was eaten each day and adjusted as the larvae increased in size. Pupation was observed every 24 h, with pupae being counted each day and transferred to small containers, with the sex confirmed following eclosion.

Whole-Animal Assay: *Drosophila*

Fly food was prepared with Jazz Mix *Drosophila* food (Fisher Scientific, Waltham, MA). Powder was mixed with dH₂O, boiled, and then placed in a 42 °C water bath to cool. Upon reaching 42 °C, 5 mL of the food was added to vials along with the compound and a dye indicator to demonstrate proper mixing. Food was allowed to cool 6 to 24 h at room temperature. Embryos were then collected and counted into groups of 50 on a bed of agar. Agar with a group of embryos on top was excised and placed into each of the food-containing vials. Two days later, the agar was removed, and unhatched embryos were counted. The vials were then monitored for pupation and eclosion rates based on the number of hatched embryos.

Results

The schematic in Figure 1 details the workflow of the high-throughput screening platform. In the first phase, the mosquito cell line 4A3A (derived from *Anopheles gambiae*) was screened with a structurally diverse chemical library containing 8000 inert small molecules (referred to as the UTSW 8K library). From this primary screen, a total of 176 generic cytostatic or cytotoxic compounds were identified. In the second phase, each was retested against the same 4A3A cell line and, in parallel, counterscreened against two different *Drosophila* lines (S2R⁺ and Kc). Note that all three of these cell lines are cultured in the same media, thereby ensuring that the differences in sensitivity trace to the biology of the cells rather than the conditions under which they are cultured. The heat map in Figure 1 illustrates compounds with activity specific for 4A3A cells. To prioritize these compounds, the stringency of the Z score thresholds was increased. In addition, legacy data on the UTSW 8K library was used to exclude compounds active against any of eight human cell lines that were previously screened. These analyses produced a panel of 16 compounds, and as shown in Supplemental Figure S1, these were qualitatively scored for cytostatic versus cytotoxic effects. However, for simplicity, this collection will hereafter be referred to as selective cytotoxins.

The panel of selective cytotoxins was newly acquired from a commercial vendor (except SW060760), and each was retested against the original mosquito line 4A3A and two *Drosophila* lines S2R⁺ and Kc. Thirteen compounds were confirmed for targeted activity. These were also empirically tested for activity in three human cell lines to affirm inferences based on legacy data (Supplemental Fig. S2; Table 1). To further prioritize these lead compounds, each was profiled for activity against additional mosquito cell cultures, including two *Anopheles gambiae* (vector for malaria) lines as well as one *Anopheles stephensi* (vector for malaria) and one *A. aegypti* (vector for dengue fever) line. Several categories of cytotoxins emerged from these assays. Three compounds produced broad activity against all mosquito lines tested, and five compounds showed narrow activity, killing more than one but not all mosquito lines tested (Fig. 2A,B). Five compounds were idiosyncratic, with activity in only 4A3A cells (Supplemental Fig. S3). Hits that were uniformly lethal to both *A. gambiae* lines were carried forward in subsequent studies.

Long-term stability is an important logistical consideration in the manufacturing and distribution of pesticides. Likewise, aqueous stability at ambient temperatures is particularly critical for efficacy in the field. Therefore, the remaining eight lead compounds (broad and narrow cytotoxicity) were tested after long-term storage for 1 mo at ambient temperatures. Of the eight lead compounds, two retained significant toxicity in both solvents (DMSO or H₂O), and a third compound showed some reduction in cytotoxicity but retained >50% activity in water (Fig. 2C; Supplemental Fig. S4). The three compounds SW120412, SW138658, and SW049753 were carried forward in subsequent studies.

The UTSW 8K library used in the primary screen is a representative subset of a larger institutional library at UT Southwestern containing 200 000 compounds (UTSW 200K library). To evaluate the potential for chemical optimization, compound families sharing core structures with each of the three lead cytotoxins were recovered from the UTSW 200K library and similarly tested for both toxicity and specificity (Fig. 3A–C). The results show that within these family groups, some but not all compounds were active. Furthermore, cytotoxicity never dissociated from species specificity. These data exclude nonspecific mechanisms of killing and also affirm the feasibility of optimizing compounds through structure-activity relationship (SAR) analysis. The corresponding core structures are shown in Figure 3D–F.

To more precisely resolve IC₅₀ values for the three lead cytotoxins seen in Figure 2C, each was assayed using an Echo 555 liquid-handling instrument to dispense half-log doses down from a top concentration of 50 μ M or 500 μ M. IC₅₀ values for the mosquito line 4A3A, two *Drosophila* lines, and two human lines are seen in Table 1. Note that all showed potent selectivity windows with IC₅₀ values representing 10- to 170-fold better activity in mosquito cells relative to the fly and human lines tested. One impressive candidate, SW137658, had an IC₅₀ of 2.86 μ M for the cell line 4A3A, but no IC₅₀ value was detected in the human cell lines tested (see also Supplemental Fig. S2).

A premise built into the platform predicts that selection for mosquito-specific toxicity in culture should enrich for compounds with targeted toxicity at the whole-animal level. To test this prediction, the three water-stable, lead cytotoxins were assayed for larvicidal activity against *Anopheles*, *Aedes*, and *Drosophila* samples. SW120412 shows striking mosquito-specific toxicity (Fig. 4), whereas the other two compounds were inactive. Furthermore, whole-animal toxicity caused by SW120412 tracked with activity seen in cultured cells (Fig. 2A) because *A. gambiae* larvae were far more sensitive than *A. aegypti* larvae.

Discussion

There is a broad consensus that eradication of mosquitoborne diseases will require new methods for controlling mosquitoes as resistance to one pesticide often yields cross-resistance to many pesticides.^{2,7} In addition, new threats such as West Nile Virus are creating epidemics in developed areas where residents are concerned that aerial spraying of pesticides could adversely affect public health and the ecosystem.¹⁴ This proof-of-principle study demonstrates that a cell-based screening strategy can identify mosquito-specific, water-stable cytotoxins, which, in at least one case, translated to targeted killing in whole-animal assays (Fig. 4). This platform offers significant advantages over whole-animal screens. For example, using at least an order of magnitude less compound, the cell-based strategy increased throughput nearly two orders of magnitude relative to Pridgeon et al.⁸ within the same time period. As a system for pesticide development, the methodical platform described here has several compelling advantages. First, the screen is intrinsically biased toward novel targets because the cultures used here are nonneuronal⁹ yet most pesticides are neurotoxins.¹⁵ Second, the capabilities enabled by this platform greatly outperform whole-animal screens, permitting us to sample compound libraries on vast scales in a fraction of the time and at a fraction of the cost. Similarly, the platform also enables efficient optimization of lead compounds through comprehensive SAR studies that would not be practical in whole-animal assays. For example, as seen in Figure 3, SAR experiments could be applied to improve the efficacy of compounds such as SW120412 or, alternatively, explore why some promising cytotoxic scaffolds fail when tested in animals. A final advantage is versatility. When conducted on a much larger scale, the platform could be easily tailored to include additional selections for desirable characteristics or to exclude unfavorable properties. For example, data from empirically testing the three human lines

was combined with legacy information on eight human lines; however, additional lines could be counterscreened to better represent a complete spectrum of tissue types. The importance of this is underscored by an indication that one of the lead compounds synergized with a synthetic retinoid to reduce the viability of two neuroblastoma cell lines.^{16–19} Likewise, other lines from beneficial insects such as the bumble bee²⁰ could be included to further limit off-target effects in the ecosystem. These features, combined with the proof-of-concept and lead compounds discovered here, offer a platform for generalized pesticide development that could be exploited to target other disease vectors as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A cell-based screening platform identifies targeted mosquito-specific cytotoxins. The workflow illustrated to the left is a high-throughput cell-based screening strategy designed to identify mosquitocidal cytotoxins. Lead compounds listed on the right were assessed for cross-species activity and long-term stability (legend below).

Figure 2.

Assessment of selective cytotoxins. (A) and (B) show activity profiles for the selective cytotoxins that were not specific to the cell line 4A3A. The data in (C) shows long-term aqueous stability of the same compounds plotted relative to frozen stocks.

Figure 3. Structure activity analysis. Compound families sharing core structures in common with each lead cytotoxin were assembled and tested. (A–C) Activities for each member of these compound families. The parental compound is indicated with an (*). (D–F) Core structure defining each family group. Activity-dependent positions are labeled as an R. Positions not associated with activity are denoted with an (*).

Figure 4.

Identification of a targeted mosquitocidal compound. All three lead compounds from Table 1 were subsequently tested for larvicidal activity in whole-animal assays. SW120412 showed impressive potency against *A. gambiae*, whereas *Drosophila* larvae were unaffected for eclosion rates, even when exposed to 200 μ M SW120412 (normalized eclosion rates of 109% and 97%).

Table 1Quantitation of Cytotoxicity of the Three Lead Compounds^a

	IC ₅₀ (μM)		
	SW120412	SW137658	SW049753
4A3A (<i>Anopheles</i>)	1.42 ± 0.52	2.86 ± 6.29	14.6 ± 7.4
Kc (<i>Drosophila</i>)	210 ± 30.6	170 ± 50	120 ± 13.5
S2R ⁺ (<i>Drosophila</i>)	240 ± 19.4	>50 μM	130 ± 2.9
HCT116 (human)	35 ± 5.15	>50 μM	205 ± 6.6
TPH1 (human)	120 ± 35.6	>50 μM	140 ± 22.8

^a Activity profiles for the lead cytotoxins were refined by quantitating IC₅₀ values (in μM) for the *Anopheles* line 4A3A, two fly lines, and two human lines. The values are at μM concentrations with corresponding standard deviations given.