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Evaluation of *Aa*DOP2 Receptor Antagonists Reveals Antidepressants and Antipsychotics as Novel Lead Molecules for Control of the Yellow Fever Mosquito, *Aedes aegypti*

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ABSTRACT

The yellow fever mosquito, *Aedes aegypti*, vectors disease-causing agents that adversely affect human health, most notably the viruses causing dengue and yellow fever. The efficacy of current mosquito control programs is challenged by the emergence of insecticide-resistant mosquito populations, suggesting an urgent need for the development of chemical insecticides with new mechanisms of action. One recently identified potential insecticide target is the *A. aegypti* D_1-like dopamine receptor, *Aa*DOP2. The focus of the present study was to evaluate *Aa*DOP2 antagonism both in vitro and in vivo using assay technologies with increased throughput. The in vitro assays revealed *Aa*DOP2 antagonism by four distinct chemical scaffolds from tricyclic antidepressant or antipsychotic chemical classes, and elucidated several structure-activity relationship trends that contributed to enhanced antagonist potency, including lipophilicity, halide substitution on the tricyclic core, and conformational rigidity. Six compounds displayed previously unparalleled potency for in vitro *Aa*DOP2 antagonism, and among these, asenapine, methiothepin, and *cis*-(*Z*)-flupenthixol displayed subnanomolar IC_{50} values and caused rapid toxicity to *A. aegypti* larvae and/or adults in vivo. Our study revealed a significant correlation between in vitro potency for *Aa*DOP2 antagonism and in vivo toxicity, suggesting viability of *Aa*DOP2 as an insecticidal target. Taken together, this study expanded the repertoire of known *Aa*DOP2 antagonists, enhanced our understanding of *Aa*DOP2 pharmacology, provided further support for rational targeting of *Aa*DOP2, and demonstrated the utility of efficiency-enhancing in vitro and in vivo assay technologies within our genome-to-lead pipeline for the discovery of next-generation insecticides.

Introduction

Mosquitoes transmit pathogens and parasites that cause diseases that adversely affect human health worldwide, including malaria, yellow fever, and dengue. Existing approaches for mosquito control have demonstrated efficacy in reducing incidences of such diseases, but are becoming inadequate due to the emergence of insecticide-resistant mosquito populations (Hemingway and Ranson, 2000; Hemingway, 2014). The need for novel mode-of-action compounds to control mosquitoes is further emphasized by the fact that it has been several decades since a new public health insecticide has been deployed to reduce the spread of vector-borne diseases (Hemingway et al., 2006).

Arthropod G protein–coupled receptors (GPCRs) mediate critical biologic processes (Hauser et al., 2006) and have emerged as potential insecticide targets (Hill et al., 2013). Molecular approaches, including genome sequencing efforts, have identified more than 100 GPCRs within the genomes of several arthropod vector species (Hill et al., 2002; Nene et al., 2007; Arensburger et al., 2010; Kirkness et al., 2010). Among the GPCR superfamily, the biogenic amine receptors are of particular interest because of their crucial roles in insect physiology and behavior (Hauser et al., 2006; Fuchs et al., 2014). For example, the biogenic amine dopamine and its receptors are implicated in a variety of arthropod behaviors, including arousal (Kume et al., 2005), locomotion (Yellman et al., 1997; Draper et al., 2007; Mustard et al., 2010), and olfactory learning (Kim et al., 2007; Riemensperger et al., 2011). It is also notable that dopamine is associated with the salivary function of vectors (Ali, 1997; Sauer et al., 2000; Šimo et al., 2011, 2014), suggesting potential roles for the mediation of pathogen acquisition and transmission during blood feeding. In *Aedes aegypti*, dopamine is also implicated in sclerotization and ovarian/egg development, as increased dopamine levels were observed in newly emerged

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ABBREVIATIONS: GPCR, G protein–coupled receptor; HEK, human embryonic kidney; HEK-*Aa*DOP2, HEK293 cells stably expressing *Aa*DOP2; HEK-hD_1, HEK293 cells stably expressing the human D_1 dopamine receptor; HTRF, homogenous time-resolved fluorescence; HTS, high-throughput screening; LY-310,762, 1-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-1,3-dihydro-3,3-dimethyl-2*H*-indol-2-one; R59-022, 6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidin-5-one; SAR, structure-activity relationship; SCH-23390, (*R*)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine.

adults and following a blood meal (Andersen et al., 2006). The central roles of dopamine systems in fundamental biologic processes offer the dopamine receptors as potential insecticide targets.

A recent study from our invertebrate receptor group supports the pursuit of D_1-like dopamine receptors (*Aa*DOP1 and *Aa*DOP2) from the yellow fever mosquito, *A. aegypti*, as targets for novel mode-of-action insecticides (Meyer et al., 2012). Specifically, *Aa*DOP2 was used as a prototypical target for a "genome-to-lead" approach for the discovery of target-based insecticides, where genomic sequence data were used to drive in vitro functional characterization of recombinant *Aa*DOP receptors in human embryonic kidney (HEK) 293 cells (Meyer et al., 2012). Following pharmacologic characterization, high-throughput screening (HTS)–amenable evaluation of pharmacologically active compounds identified *Aa*DOP2 antagonists that display significant in vivo toxicity to mosquito larvae (Meyer et al., 2012), supporting the validity of targeting *Aa*DOP2 for *A. aegypti* control.

The present study entailed a robust follow-up pharmacologic analysis of *Aa*DOP2 antagonists identified in a small-molecule screen of the $LOPAC_{1280}$ library (Meyer et al., 2012). To accomplish this, we developed an HTS-amenable cell-based assay that enabled an in-depth study of *Aa*DOP2 antagonism by tricyclic antidepressants and structurally related compounds. Several of these compounds demonstrated enhanced potency for in vitro *Aa*DOP2 antagonism and greater efficacy for larval death in mosquito bioassays. Importantly, we provided evidence that several *Aa*DOP2 antagonists caused toxicity in adult *A. aegypti*. Furthermore, we improved upon our previously described genome-to-lead pipeline via implementation of efficiency-enhancing in vivo assay technologies.

Materials and Methods

Cis-(*Z*)-flupenthixol, clozapine, mianserin, nortriptyline, imipramine, protriptyline, norclomipramine, pirenperone, desipramine, haloperidol, trazodone, fluoxetine, fluvoxamine, buspirone, (+)-butaclamol, amoxapine, amitriptyline, chlorpromazine, doxepin, loratadine, ketotifen, chlorprothixene, loxapine, cyproheptadine, asenapine, diphenhydramine, ritanserin, ketanserin, risperidone, 3-isobutyl-1-methylxanthine, G418, and Dulbecco's modified Eagle's medium were purchased from Sigma-Aldrich (St. Louis, MO). Amperozide, methiothepin, clomipramine, SCH-23390 [(*R*)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine], LY-310,762 (1-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-1,3-dihydro-3,3-dimethyl-2*H*-indol-2-one), R59-022 (6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidin-5-one), and tomoxetine were purchased from Tocris Bioscience (Ellisville, MO). Benztropine was purchased from Enzo Life Sciences (Farmingdale, NY). The antibiotic-antimycotic 100× solution was purchased from Life Technologies (Grand Island, NY). FetalClone I serum, bovine calf serum, HEPES, and Hanks' balanced salt solution were purchased from Hyclone (Logan, UT). The homogenous time-resolved fluorescence (HTRF) cAMP kit was purchased from Cisbio Bioassays (Bedford, MA).

Cisbio HTRF cAMP Dynamic 2 Cell-Based Assay. HEK293 cells stably expressing *Aa*DOP2 (HEK-*Aa*DOP2) or the human D_1 dopamine receptor (HEK-hD_1) were maintained and cryogenically frozen as previously described (Meyer et al., 2012). To prepare for pharmacologic analysis, cells were thawed and resuspended in assay buffer (Hanks' balanced salt solution, 20 mM HEPES, 0.1% fatty acid–free bovine serum albumin). To remove cryogenic freezing media, cell suspensions were centrifuged at 500*g* for 5 minutes, followed by aspiration of the supernatant. Cell pellets were resuspended in assay buffer and seeded into 384-well plates (CulturPlate-384; PerkinElmer, Waltham, MA) at 2000–2500 cells per well and incubated at 37°C and 5% CO_2 for 1 hour. Test compounds were added using a 384-well pin tool (V&P Scientific, San Diego, CA). A MultiFlo (BioTek, Winooski, VT) low-volume bulk reagent dispenser was used to dispense 3 μM dopamine (in assay buffer containing 500 μM 3-isobutyl-1-methylxanthine and 0.02% ascorbic acid) to activate *Aa*DOP2. Drug stimulation was carried out at room temperature for 1 hour. Cells were lysed by sequential addition of cAMP-d_2 and anti-cAMP cryptate conjugate, both diluted 1:39 in lysis buffer, and were incubated at room temperature for 1 hour. Time-resolved fluorescence resonance energy transfer was measured with a lag time of 100 microseconds and integration time of 300 microseconds using a Synergy4 (BioTek) fluorescence plate reader with a 330/80-nm excitation filter and emission filters of 620/10 and 665/8 nm. Sensitivity parameters were set by reading the cAMP standard curve using the autosensitivity setting. All experimental conditions were read using sensitivity settings obtained for the cAMP standard curve. Cellular cAMP concentrations were estimated in GraphPad Prism (GraphPad Software, La Jolla, CA) by applying the 620/665-nm fluorescence ratio values to a standard curve of known cAMP concentrations.

Cyclic AMP measurements in HEK293 cells stably expressing the human D_1 dopamine receptor were performed as described earlier, but 500 nM dopamine was used to stimulate cAMP accumulation.

In Vivo *A. aegypti* Larval Screen. Test compounds were evaluated for in vivo toxicity in bioassays against L3 stage *A. aegypti* larvae in a double-blind manner. In brief, compounds were resuspended in water and added to wells of a 24-well plate (BD Bioscience, San Jose, CA) in duplicate, with each well containing five *A. aegypti* larvae in 1-ml total volume to achieve a final concentration of 400 μM per well (see Supplemental Fig. 1 for illustrations of the assay format). Plates were incubated at 22°C, and the assay was scored for larval mortality at 24, 48, and 72 hours. Larvae unresponsive to gentle tapping of the plate or touch with a sterile probe were scored as dead.

***A. aegypti* Adult Concentration-Response Curves.** Test compounds were dissolved in deionized water to a 200 mM stock concentration and serially diluted in *Aedes* saline (Hayes, 1953) to achieve a dose range of 0.25–20 mM. Four-day-old *A. aegypti* adult females [average wing length of 3.4 mm, measured as described by Briegel (1990)] were anesthetized on ice, and groups of 20 females were injected with the indicated amounts of test compounds (0.5 μl per mosquito) or *Aedes* saline alone (control) using a pulled glass capillary needle. Additional uninjected mosquito controls were also included. Mosquitoes were housed in 10-cm diameter × 20-cm height paper coffee cup cages with lace screens (secured with rubber bands) and maintained at 75% humidity with 10% sucrose provided ad libitum via a cotton wick (see Supplemental Fig. 2 for illustrations of injections and mosquito housing). Observations of mortality were made daily for up to 4 days post-treatment. Mosquitoes were scored as "dead" if no movement was observed and confirmed by no response to a gentle touch of the legs with a metal probe. When observed at any time point, moribund adult mosquitoes (i.e., insects incapable of standing, walking, or flying) were scored as dead. At the 24-hour time point, and to a lesser extent at the 48-hour time point, we observed a percentage of the adult mosquito population that was moribund. These mosquitoes did not recover and died by assay endpoint. The moribund phenotype was negligible at 96 hours (less than 1% of the adult population for any replicate dose). LD_{50} values for test compounds injected into adult mosquitoes were calculated by nonlinear regression using the sigmoidal dose-response equation in the GraphPad Prism software.

Results

In Vitro Evaluation of *Aa*DOP2 Antagonism. Our previous studies indicated potential value in pursuing *Aa*DOP2 in a target-first approach for developing new insecticides against *A. aegypti* (Meyer et al., 2012). We also demonstrated

the success of utilizing a heterologous cell model, where recombinant *Aa*DOP2 receptors are expressed in HEK293 cells (HEK-*Aa*DOP2) for identification and pharmacologic evaluation of novel *Aa*DOP2 ligands (Meyer et al., 2012). To improve upon our genome-to-lead pipeline for novel insecticide discovery, HEK-*Aa*DOP2 cells were used to develop a cell-based assay that enabled rapid and efficient study of receptor antagonists. The Cisbio HTRF cAMP Dynamic 2 detection methodology was chosen as the assay platform, allowing for the direct detection of cAMP in a 384-well format, and initial experiments were focused on validating cAMP responses to dopamine stimulation using this assay format. As *Aa*DOP2 is a Gα_s-coupled D_1-like dopamine receptor, stimulation with dopamine results in an enhanced level of cAMP (Meyer et al., 2012). As expected, dopamine treatment displayed a concentration-dependent enhancement of cAMP accumulation with an EC_{50} of 950 ± 190 nM ($n = 5$). The EC_{50} of dopamine was similar to that determined in the previous [^{3}H]cAMP-based quantification method (Meyer et al., 2012). Furthermore, the potency of amitriptyline (the prototypical mosquito-toxic *Aa*DOP2 antagonist) for inhibition of dopamine-stimulated cAMP in the HEK-*Aa*DOP2 cells was similar to that previously reported (Tables 1 and 2) (Meyer et al., 2012; Hill et al., 2013), demonstrating suitability of the HTRF cAMP detection technology for high-throughput cell-based pharmacologic studies of *Aa*DOP2.

Our previous screen of the $LOPAC_{1280}$ library identified 51 active compounds as *Aa*DOP2 antagonists, including several tricyclic antidepressants (Meyer et al., 2012). Importantly, two tricyclic antidepressant compounds, amitriptyline and doxepin, cause significant mortality of mosquito larvae in whole-organism bioassays, suggesting the potential of tricyclic antidepressants as insecticide lead compounds (Meyer et al., 2012). To gain an understanding of the chemical features that are important for *Aa*DOP2 antagonist activity, pharmacologic evaluation of additional small molecules that are structurally related to the tricyclic leads was carried out in the cell-based assay described earlier. Specifically, nine tricyclic antidepressants and five antidepressant compounds lacking a tricyclic core were studied for their ability to antagonize the cAMP accumulation in response to dopamine treatment (3 μM) in HEK-*Aa*DOP2 cells. All nine tricyclic compounds displayed concentration-dependent antagonist activity against *Aa*DOP2, with IC_{50} values less than 3 μM, whereas compounds representing other classes of antidepressants displayed less than 10% inhibition at 3 μM (Fig. 1; Table 1).

TABLE 1

Evaluation of antidepressant compounds from distinct classes for antagonism of the *Aa*DOP2 receptor

The effect of various concentrations of antidepressant compounds was tested for inhibition of 3 μM dopamine-stimulated cAMP in HEK-*Aa*DOP2 receptor cells. Data represent the mean ± S.E.M. IC_{50} values for at least three independent experiments.

Compound	IC_{50} ± S.E.M.	Chemical Class
	nM	
(+)-Butaclamol	260 ± 32	DR antagonist
Amitriptyline	5.1 ± 1.2	TCA
Amoxapine	20 ± 8.4	TeCA
Atomoxetine	No inhibition[a]	NRI
Clomipramine	56 ± 18	TCA
Desipramine	3300 ± 600	TCA
Doxepin	20 ± 6.2	TCA
Fluoxetine	No inhibition[a]	SSRI
Fluvoxamine	No inhibition[a]	SSRI
Imipramine	360 ± 34	TCA
Norclomipramine	670 ± 35	TCA
Nortriptyline	140 ± 50	TCA
Protriptyline	600 ± 250	TCA
SCH-23390	1300 ± 340	D_1DR antagonist
Trazodone	No inhibition[a]	SARI
Venlafaxine	No inhibition[a]	SNRI

D_1DR, selective D_1-like dopamine receptor antagonist; DR antagonist, non-selective dopamine receptor antagonist; NRI, norepinephrine reuptake inhibitor; SARI, serotonin antagonist and reuptake inhibitor; SNRI, serotonin and norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; TeCA, tetracyclic antidepressant.

[a]Less than 10% inhibition at 3 μM compound.

TABLE 2

Pharmacologic characterization of compounds for antagonist activity against the *Aa*DOP2 receptor

The effect of various concentrations of compounds was tested for inhibition of 3 μM dopamine-stimulated cAMP in HEK-*Aa*DOP2 receptor cells. The data represent the mean ± S.E.M. IC_{50} values for at least three independent experiments.

Compound	IC_{50} ± S.E.M.
	nM
(+)-Butaclamol	160 ± 31
Amitriptyline	7.2 ± 1.2
Amperozide	570 ± 110
Aripiprazole	6500 ± 770
Asenapine	0.30 ± 0.06
Benztropine	340 ± 41
Chlorpromazine	17 ± 0.88
Chlorprothixene	1.2 ± 0.39
Cis-(*Z*)-flupenthixol	0.35 ± 0.07
Clozapine	14 ± 2.9
Cyproheptadine	6.5 ± 1.9
Diphenhydramine	7500 ± 2800
Haloperidol	4300 ± 1000
Ketanserin	3200 ± 360
Ketotifen	750 ± 180
Loratadine	18,000 ± 1800
Loxapine	5.9 ± 1.4
LY-310,762	3000 ± 820
Methiothepin	0.25 ± 0.05
Mianserin	130 ± 24
Olanzapine	11 ± 2.2
Pirenperone	680 ± 98
R59-022	53 ± 13
Risperidone	150 ± 41
Ritanserin	500 ± 110

To identify novel *Aa*DOP2 antagonists with chemical structures distinct from the tricyclic antidepressant ring scaffold, we evaluated concentration-dependent effects of a suite of additional active compounds identified in our previous small-molecule screen (Meyer et al., 2012) together with structurally related compounds, enabling an initial in vitro structure-activity relationship (SAR) analysis. As performed earlier, test compounds were studied for their ability to modulate dopamine-stimulated (3 μM) cAMP accumulation in HEK-*Aa*DOP2 cells (Table 2). Interestingly, six compounds were more potent antagonists than the prototypical *Aa*DOP2 antagonist amitriptyline (Table 2). Furthermore, asenapine, methiothepin, and *cis*-(*Z*)-flupenthixol displayed subnanomolar IC_{50} values for inhibition of dopamine-stimulated cAMP in HEK-*Aa*DOP2 cells (Table 2).

Pharmacologic selectivity for the targeted insect over humans and other animals is a critical attribute of potential insecticides. To address this concern, several of the most potent *Aa*DOP2 antagonists were evaluated for antagonist activity in HEK293 cells stably expressing the human D_1 dopamine receptor (HEK-hD_1 cells) and compared with the hD_1 antagonist, SCH-23390. Each compound inhibited 500 nM dopamine-stimulated cAMP in

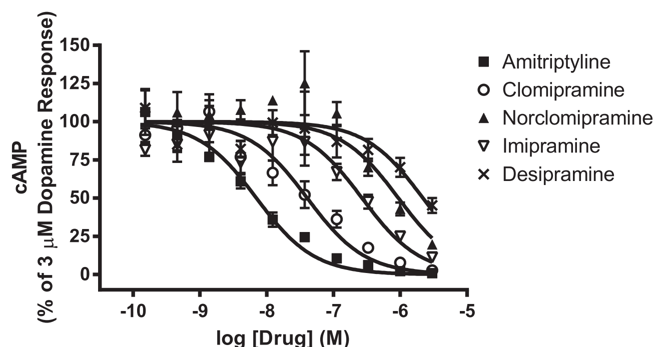

Fig. 1. Concentration-response curves for selected *Aa*DOP2 antagonists. Test compounds were evaluated for the ability to inhibit dopamine (3 μM)-stimulated cAMP in HEK-*Aa*DOP2 cells. Data points represent the mean ± S.E.M. for at least three independent experiments.

the HEK-hD_1 cells and displayed IC_{50} values between 19 and 13,000 nM (Table 3). However, in contrast to the hD_1-selective antagonist SCH-23390, all of these compounds were more potent antagonists of *Aa*DOP2 than hD_1, suggesting potential species-selective pharmacologic profiles for these compounds.

In Vivo Toxicity of *Aa*DOP2 Antagonists: Effects on *A. aegypti* Larvae. An important second step in our insecticide discovery effort was the evaluation of the in vivo activity of compounds identified and characterized in the cell-based in vitro studies. We developed an *A. aegypti* larval screen that can be performed in a 24-well plate format, allowing rapid assessment of in vivo toxicity for compounds identified as potent antagonists in the in vitro studies. This assay was designed to also enable evaluation of speed-to-kill and support prioritization of compounds for further study. Twenty-five compounds were tested using this approach (Table 4), and 10 compounds [asenapine, chlorpromazine, benztropine, methiothepin, *cis*-(*Z*)-flupenthixol, chlorprothixene, loxapine, mianserin, amperozide, and clomipramine) caused 70–100% larval mortality within 24 hours post-treatment. These compounds were faster-acting and caused greater mortality of mosquito larvae at the 24-hour treatment time point than our previously identified lead compound for insecticide development, amitriptyline. Notably, asenapine, chlorpromazine, and amperozide caused greater than 70% mortality of the mosquito population within 30 minutes, and *cis*-(*Z*)-flupenthixol, chlorprothixene, mianserin, loxapine, and methiothepin caused greater than 70% mortality within 3 hours (data not shown). We also identified five compounds with moderate mosquito toxicity (i.e., 40–70% mortality at 24 hours postexposure) and nine compounds with limited or no toxicity to mosquito larvae (i.e., 0–40% mortality at 24 hours) (Table 4). The in vivo larval mortality data show a significant correlation with in vitro potency values for antagonism of dopamine-stimulated cAMP in HEK-*Aa*DOP2 cells ($r = -0.770$, $n = 25$, $P > 0.0001$; Fig. 2), providing an important line of evidence that *Aa*DOP2 antagonism is linked to larval toxicity.

In Vivo Toxicity of *Aa*DOP2 Antagonists: Effects on Adult *A. aegypti*. Toxicity to adult female *A. aegypti* is considered an important property of any lead molecule because only adult female mosquitoes are responsible for the transmission of disease-causing agents. Therefore, we developed an adult *A. aegypti* assay to evaluate the effects of *Aa*DOP2 antagonists following introduction to the insect hemocoel via microinjection. Four of the most potent in vitro and/or most efficacious compounds in the larval bioassay were assessed for toxicity (LD_{50}) and speed-to-kill in adult mosquito bioassays (Fig. 3; Table 5). All compounds tested caused dose-dependent toxicity to adult *A. aegypti* and were capable of providing 100% mortality at all time points, whereas <6% mortality was observed for the saline-injected and uninjected controls throughout the 96-hour experiments. *Cis*-(*Z*)-flupenthixol was the most potent compound, having an LD_{50} of 1.26 nmol/mosquito following 24-hour exposure (Fig. 3). Chlorpromazine and *cis*-(*Z*)-flupenthixol became more potent over the course of the 4-day assay, as LD_{50} values decreased by ~2- to 3-fold from the 24- to 96-hour time points for these compounds. In contrast, the LD_{50} values for amitriptyline and amperozide remained relatively stable over the same treatment duration, suggesting that these compounds reach their maximum potency earlier than chlorpromazine and *cis*-(*Z*)-flupenthixol (Table 5).

Discussion

The active ingredients of the major existing neurotoxic classes of insecticides target acetylcholinesterases (organophosphates and carbamates), GABA receptors (organochlorines), and sodium channels (pyrethroids) within insect nervous systems (Hemingway and Ranson, 2000). Continued efficacy of modern commercial insecticides is threatened by the development of insect populations that are resistant to these chemicals (Hemingway and Ranson, 2000), emphasizing the urgency of developing insecticides with new modes of action. Our recent

TABLE 3
Assessment of compound potency for human D_1 receptor antagonism
The effect of various concentrations of compounds was tested for inhibition of 500 nM dopamine-stimulated cAMP in HEK-hD_1 cells. The data represent the mean ± S.E.M. IC_{50} values for four independent experiments.

Compound	IC_{50} ± S.E.M.	Relative Fold Selectivity (*Aa*DOP2/hD_1)
	nM	
Amitriptyline	1100 ± 110	170
Amperozide	13000 ± 680	23
Asenapine	150 ± 11	500
Chlorpromazine	750 ± 80	44
Chlorprothixene	49 ± 8.5	41
Cis-(*Z*)-flupenthixol	19 ± 1.7	54
Cyproheptadine	1400 ± 160	220
Doxepin	2500 ± 240	130
Loxapine	300 ± 31	51
Methiothepin	83 ± 9.0	330
SCH-23390	1.2 ± 0.20	0.0009

TABLE 4
In vivo toxicity of test compounds to *A. aegypti* larvae
Data represent the mean ± S.E.M. of three independent experiments.

Compound	Larval Mortality		
	24 h	48 h	72 h
	%		
Amitriptyline	63 ± 20	87 ± 7	93 ± 3
Amperozide	93 ± 7	93 ± 7	93 ± 7
Asenapine	100 ± 0	100 ± 0	100 ± 0
Benztropine	100 ± 0	100 ± 0	100 ± 0
Chlorpromazine	100 ± 0	100 ± 0	100 ± 0
Chlorprothixene	87 ± 9	93 ± 7	100 ± 0
Cis-(*Z*)-flupenthixol	100 ± 0	100 ± 0	100 ± 0
Clomipramine	70 ± 21	93 ± 3	93 ± 3
Desipramine	30 ± 25	40 ± 30	43 ± 28
Diphenhydramine	63 ± 12	77 ± 9	83 ± 9
Fluoxetine	43 ± 30	53 ± 24	53 ± 24
Fluvoxamine	27 ± 22	33 ± 28	43 ± 24
Imipramine	53 ± 26	63 ± 20	80 ± 12
Ketanserin	0 ± 0	0 ± 0	0 ± 0
Loxapine	97 ± 3	100 ± 0	100 ± 0
LY-310,762	0 ± 0	3 ± 3	3 ± 3
Methiothepin	100 ± 0	100 ± 0	100 ± 0
Mianserin	97 ± 3	97 ± 3	97 ± 3
Norclomipramine	40 ± 31	63 ± 19	70 ± 15
Nortriptyline	43 ± 28	63 ± 19	73 ± 15
Pirenzepine	0 ± 0	0 ± 0	0 ± 0
Protriptyline	37 ± 12	43 ± 13	53 ± 23
SCH-23390	3 ± 3	23 ± 12	47 ± 23
Tomoxetine	20 ± 15	30 ± 20	30 ± 20
Venlafaxine	3 ± 3	7 ± 7	13 ± 9
Control (water only)	0 ± 0	1 ± 1	3 ± 1

study identified the dopamine receptors of *A. aegypti* as potential targets for yellow fever mosquito control (Meyer et al., 2012). Specifically, larval toxicity was observed for two tricyclic antidepressant compounds (amitriptyline and doxepin) that display *Aa*DOP2 antagonism (Meyer et al., 2012).

To better understand the chemical basis of *A. aegypti* toxicity observed in vivo, compounds with activity profiles similar to amitriptyline and doxepin at human targets (i.e., GPCRs and/or biogenic amine transporters) were evaluated for in vitro *Aa*DOP2 modulation and in vivo efficacy in larval bioassays. Several known GPCR-targeting ligands, including tricyclic antidepressants and antipsychotics, demonstrated potent *Aa*DOP2 antagonism and insecticide activity. However,

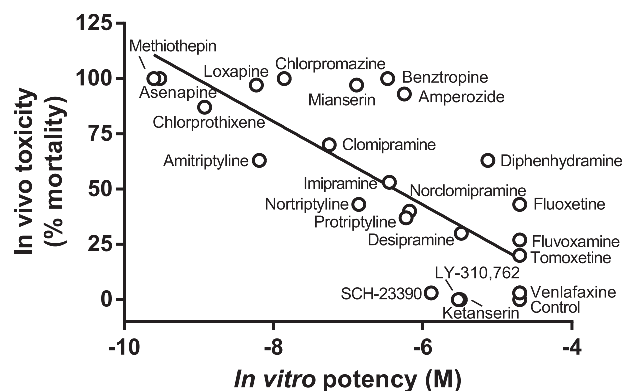

Fig. 2. Correlation analysis of test compounds evaluated for in vitro potency (IC_{50} values in HEK-*Aa*DOP2 cells) and in vivo toxicity (percent mortality of *A. aegypti* L3-stage larvae following 24-hour treatment). The in vitro potency values for compounds that provided less than 10% inhibition of dopamine-stimulated cAMP in HEK-*Aa*DOP2 cells were set to 20 μM.

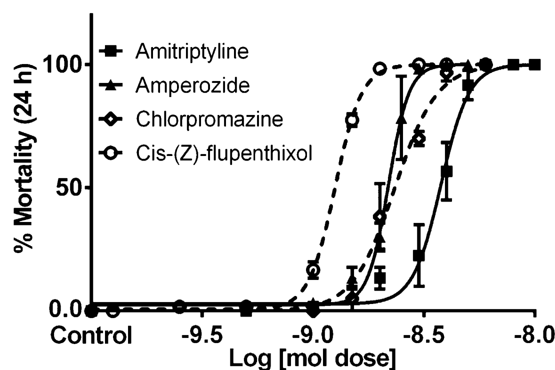

Fig. 3. Concentration-response curves of adult *A. aegypti* female mortality 24 hours after injection with *Aa*DOP2 antagonists. Each data point represents the mean ± S.E.M. for three independent experiments. No mortality was observed in saline-injected or uninjected controls at the 24-hour time point.

compounds from other antidepressant classes (e.g., selective serotonin reuptake inhibitors and selective norepinephrine reuptake inhibitors) were largely inactive, suggesting GPCR modulation, rather than biogenic amine transporters, as a contributing mechanism for the observed larval toxicity. Further validating *Aa*DOP2 as a viable insecticide target, our data revealed a significant correlation between the in vitro potency of *Aa*DOP2 antagonists and the toxicity of these compounds to mosquito larvae in vivo (Fig. 2). However, it should be noted that benztropine and amperozide, which caused rapid and high larval mortality (Table 4), had somewhat moderate in vitro potency at *Aa*DOP2 (IC_{50} values of 340 ± 41 and 570 ± 110 nM, respectively). Amperozide and benztropine interact with several different mammalian GPCR families (U'Prichard et al., 1977; Kanba and Richelson, 1984; Bolden et al., 1992; Arnt and Skarsfeldt, 1998), suggesting the possibility that modulation of additional *A. aegypti* GPCRs could contribute to the in vivo toxicity of these compounds. Alternatively, such differences between the in vitro potency and the magnitude of in vivo toxicity for a given compound may reflect complex factors that impact in vivo insecticidal activity, including differences in the physicochemical properties of compounds that affect absorption through the insect cuticle, dissemination through insect tissues to the target site, and detoxification by insect gut and hemolymph enzymes. Nonetheless, the correlation between the in vitro potencies for *Aa*DOP2 antagonism and larval toxicities suggests that optimizing compounds for potency and selectivity in vitro may be an efficient way to identify and prioritize new lead compounds.

The in vitro evaluation of the chosen compounds for modulation of *Aa*DOP2 provided preliminary insight into the relationship between chemical structure and the potency of *Aa*DOP2 antagonism. One SAR trend suggests that conformational rigidity contributes to the potency of *Aa*DOP2 antagonists. For example, compounds with 6- or 7-membered central rings were generally the most potent *Aa*DOP2 antagonists (Supplemental Fig. 3; Tables 1 and 2). However, the moderate potency of R59-022, risperidone, benztropine, and amperozide (IC_{50} values ranging from 53 to 570 nM) indicates that the central ring is not essential for antagonist activity. Benztropine was ~22-fold more potent than diphenhydramine, suggesting that conformational control of the amine moiety also contributes to the potency of compounds with no central ring (Fig. 4A). Another SAR trend

TABLE 5

Toxicity of injected *Aa*DOP2 antagonists to 4-day-old adult female *A. aegypti*

LD_{50} values (nanomole per mosquito) were calculated from dead and moribund mosquitoes, and represent the mean ± S.E.M. of three independent experiments. The average percent mortality was less than 6% for both injected and uninjected controls throughout the experiment.

Compound	24 h	48 h	72 h	96 h
Amitriptyline	3.78 ± 0.02	3.39 ± 0.02	3.09 ± 0.02	3.06 ± 0.02
Amperozide	2.19 ± 0.03	1.98 ± 0.02	1.92 ± 0.07	1.92 ± 0.03
Chlorpromazine	2.34 ± 0.02	1.97 ± 0.02	1.30 ± 0.03	1.27 ± 0.02
Cis-(*Z*)-flupenthixol	1.26 ± 0.01	0.67 ± 0.02	0.46 ± 0.03	0.42 ± 0.02

suggested that greater lipophilicity may enhance *Aa*DOP2 antagonist potency, as was observed by the ~3-fold greater potency of amitriptyline over doxepin (Fig. 4B). Furthermore, ligands with tertiary amines (clomipramine, imipramine, amitriptyline, and loxapine) were ~5- to 100-fold more potent than the secondary amine analogs of these compounds (norclomipramine, desipramine, nortiptyline, and amoxapine, respectively), demonstrating the influence of amine state on *Aa*DOP2 antagonist potency (Fig. 4C; Supplemental Fig. 3). Also, clomipramine and norclomipramine displayed ~5- to 6-fold more potent IC_{50} values than imipramine and desipramine, respectively, suggesting that halide substituents of aromatic rings within the tricyclic core can increase the potency of the identified antagonists (Fig. 4C). Enhancements in *Aa*DOP2 antagonist potency were also apparent when considering combinations of chemical properties, such as lipophilicity and halide substitution (Fig. 4C, clomipramine versus desipramine) or lipophilicity and conformational rigidity (Fig. 4D). The chemical scaffolds identified earlier and their key structural features that contribute to *Aa*DOP2 antagonism may be used to guide further lead optimization studies.

The in vitro and in vivo data presented here support the hypothesis that targeting GPCR-mediated processes is a viable strategy for identifying insecticidal compounds. However, a major challenge associated with this approach is the development of ligands that are selective for disruption of biologic activity in *A. aegypti* but not in humans or other higher eukaryotes. To date, all reports indicate that compounds that display both *Aa*DOP2 antagonism and in vivo efficacy are also known to have biologic activity in humans. Our studies identified compounds that are highly selective for targeting *Aa*DOP2 receptors over the human D_1 dopamine receptor (Table 3), but antipsychotics and tricyclic antidepressants potently interact with other families of mammalian GPCRs, including histamine, serotonin, adrenergic, and muscarinic receptors (Cusack et al., 1994; von Coburg et al., 2009). The development of ligands that selectively target biologic activity in *A. aegypti* over humans and other animals could potentially be addressed by using cell-based in vitro assays to screen against panels of human GPCRs. Also, virtual or in silico screening methods are emerging as promising approaches for the study of GPCR modulators (Shoichet and Kobilka, 2012). Such computational methodologies for lead optimization of antipsychotics and tricyclic antidepressants are strengthened by recently reported human GPCR crystal structures from histamine (Shimamura et al., 2011), serotonin (Wang et al.,

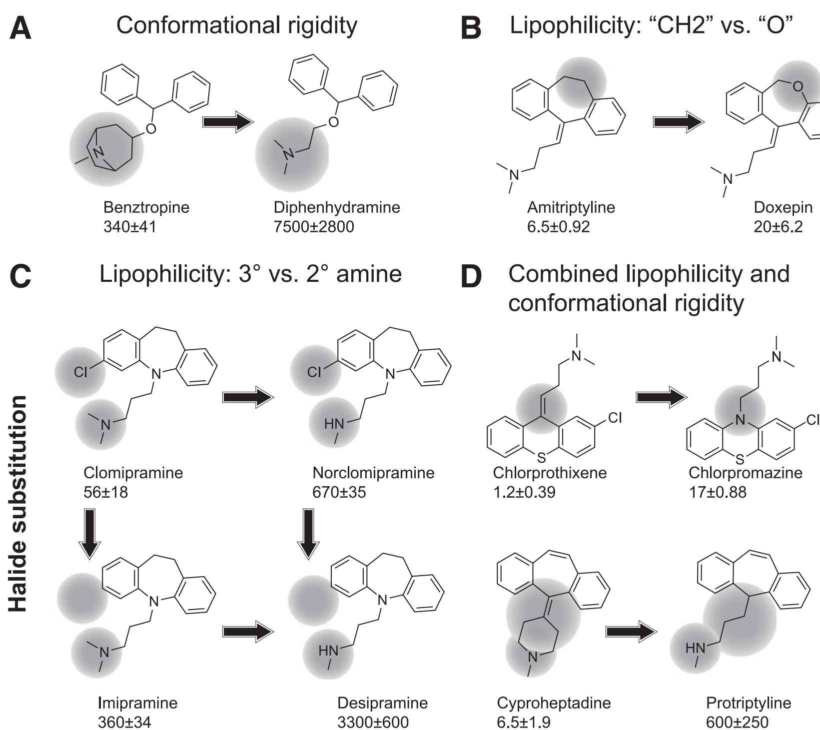

Fig. 4. (A–D) Structure-activity relationship trends for *Aa*DOP2 receptor antagonists. Compound names and in vitro IC_{50} values (nanomolar) for *Aa*DOP2 antagonism were included.

2013), dopamine (Chien et al., 2010), adrenergic (Rasmussen et al., 2007; Warne et al., 2008), and muscarinic (Haga et al., 2012; Kruse et al., 2012) receptor families. The combination of these in vitro and in silico approaches is expected to provide insight into the molecular determinants of selectivity for *Aa*DOP2 versus human GPCRs, and may ultimately produce mosquito GPCR-selective small molecules.

Pharmacologic selectivity considerations are multifold, as ligand selectivity for *Aa*DOP2 receptors over nontarget insects (e.g., honeybees), in addition to selectivity over human GPCRs, is also paramount. Pharmacologic screening panels can be assembled for invertebrate targets to better understand ligand pharmacology at these receptors. For example, cross-species comparative pharmacologic studies of invertebrate dopamine receptor modulation can be expanded to include GPCRs from nontarget insects. Furthermore, upon genome mining and cloning of additional biogenic amine receptors (in addition to *Aa*DOP1 and *Aa*DOP2), *Aa*DOP2 antagonists can be screened for modulation of other *A. aegypti* GPCRs, including muscarinic acetylcholine, serotonin, and octopamine/tyramine receptors (Nene et al., 2007). These pharmacologic efforts are expected to provide a deeper understanding of small-molecule modulation of invertebrate GPCRs, and may ultimately allow for target-based pesticide discovery efforts related to other pest arthropods.

Here we report significant advancements and modifications to our genome-to-lead insecticide discovery pipeline (Meyer et al., 2012). Incorporation of an in vitro HTRF assay enabled efficient in vitro pharmacologic evaluation and SAR profiling, while offering several advantages over the previously used CRE-mediated luciferase reporter assay for HTS. Direct measurement of cAMP eliminates false positives associated with CRE-luciferase reporter assays that include cAMP/protein kinase A–independent modulation of CRE-mediated transcription (George et al., 1997; Hill et al., 2001) or direct modulation of luciferase (Thorne et al., 2010). Furthermore, the HTRF screening platform was robust enough to support future HTS of small molecules for *Aa*DOP2 antagonist activity in a 384-well format in singlet (i.e., $Z' > 0.5$; J. Conley, C. Hill, and V. Watts, unpublished observations), enabling sufficient throughput to carry out the in vitro pharmacologic profiling proposed earlier. The HTRF screening platform also provides flexibility, as it can be used to detect modulation of additional downstream GPCR signaling pathways, including extracellular signal-regulated kinase 1/2 and Ca^{2+}/inositol trisphosphate (Degorce et al., 2009). Other improvements to our established insecticide discovery pipeline for small-molecule modulators of *Aa*DOP2 (Meyer et al., 2012) include the enhanced-throughput larval mosquito bioassay (Table 4) to rapidly assess larval toxicity and the utilization of an injection assay to evaluate toxicity in adult *A. aegypti*.

In addition to the antipsychotic and tricyclic antidepressant lead optimization and GPCR profiling studies suggested earlier, HTS of diverse small-molecule libraries for the identification of *Aa*DOP2 modulators with novel chemical scaffolds may also be a fruitful endeavor. Especially enticing is the possibility of screening for allosteric modulators of *Aa*DOP2 receptors, as drug discovery campaigns targeting multiple human GPCRs have identified allosteric modulators with unmatched specificity and selectivity (Conn et al., 2009; Wootten et al., 2013). Allosteric modulators are attractive because the orthosteric sites (i.e., the sites of endogenous ligand binding) are largely conserved between the human D_1 and *Aa*DOP2, but our published studies suggest that there are opportunities to exploit allosteric sites in the intracellular and extracellular loops where sequence similarity between species is reduced (Meyer et al., 2012; Hill et al., 2013). Our emerging understanding of the chemical basis of *Aa*DOP2 receptor antagonism, together with advancements in assay throughput, suggest that the diverse molecular approaches described earlier can be combined to expedite the discovery of novel ligands that selectively modulate GPCRs of target insects.

The present study describes the in vitro pharmacologic characterization and in vivo efficacy of several *Aa*DOP2 antagonists and demonstrates improvements upon our "genome-to-lead" pipeline (Meyer et al., 2012). Specifically, we report the characterization of compounds with unparalleled in vitro potency for *Aa*DOP2 inhibition and improved efficacy for *A. aegypti* larval toxicity, and demonstrate toxicity of these compounds to adult mosquitoes. Collectively, our findings provided a major advancement in the development of invertebrate GPCR-targeting technology for novel mode-of-action insecticides.

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Authorship Contributions

Participated in research design: Conley, Hill, Meyer, Nuss, Watts.

Conducted experiments: Conley, Doyle, Meyer, Nuss.

Contributed new reagents or analytic tools: Hill, Watts.

Performed data analysis: Conley, Doyle, Hill, Meyer, Nuss, Savinov, Watts.

Wrote or contributed to the writing of the manuscript: Conley, Hill, Meyer, Nuss, Savinov, Watts.

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