# Evaluation of *Aa*DOP2 receptor antagonists reveals antidepressants and antipsychotics as novel lead molecules for control of the yellow fever mosquito, *Aedes aegypti*

**Jason M. Conley, Jason M. Meyer, Andrew B. Nuss, Trevor B. Doyle, Sergey N. Savinov, Catherine A. Hill, and Val J. Watts**

Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, USA (JMC, TBD, VJW)

Department of Entomology, Purdue University, West Lafayette, IN, USA (JMM, ABN, CAH)

Bindley Bioscience Center, Discovery Park, Purdue University, West Lafayette, IN, USA (SNS)

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Corresponding Author:

Val J. Watts

575 Stadium Mall Dr.

West Lafayette, IN 47097

765-496-3872 (office)

765-494-1414 (fax)

wattsv@purdue.edu (email)

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Abbreviations:

CRC: Concentration-response-curve

DMEM: Dulbecco's modified Eagle's medium

GPCR: G protein-coupled receptor

HBSS: Hank's balanced salt solution

HTRF: Homogenous time-resolved fluorescence

HTS: High-throughput screening

IBMX: 3-isobutyl-1-methylxanthine

SAR: Structure-activity relationship

TR-FRET: Time-resolved fluorescence resonance energy transfer

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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 are 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* D1-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 (SAR) 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 sub-nanomolar $IC_{50}$ 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, 2014; Hemingway and Ranson, 2000). 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 biological 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 (Arensburger et al., 2010; Hill et al., 2002; Kirkness et al., 2010; Nene et al., 2007). Among the GPCR superfamily, the biogenic amine receptors are of particular interest because of their crucial roles in insect physiology and behavior (Fuchs et al., 2014; Hauser et al., 2006). For example, the biogenic amine, dopamine, and its receptors are implicated in a variety of arthropod behaviors including arousal (Kume et al., 2005), locomotion (Draper et al., 2007; Mustard et al., 2010; Yellman et al., 1997), and olfactory learning (Kim et al., 2007; Riemensperger et al., 2011). It is also notable that dopamine is associated with salivary function of vectors (Ali, 1997; Sauer et al., 2000; Simo et al., 2014; Simo et al., 2011), 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 adults and also following a blood meal (Andersen et al., 2006). The central roles of dopamine systems in fundamental biological processes offer the dopamine receptors as potential insecticide targets.

A recent study from our invertebrate receptor group supports the pursuit of D1-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 utilized 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 HEK293 cells (Meyer et al., 2012). Following pharmacological 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 pharmacological 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 to 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

*Materials*

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 (IBMX), G418, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO). Amperozide, methiothepin, clomipramine, SCH-23390, LY-310,762, R59-022, and tomoxetine were purchased from Tocris Bioscience (Ellisville, MO). Benztropine was purchased from Enzo Life Sciences (Farmingdale, NY). The antibiotic-antimycotic 100x solution was purchased from Life Technologies (Grand Island, NY). FetalClone I serum, bovine calf serum, HEPES, and Hank's balanced salt solution (HBSS) were purchased from Hyclone (Logan, UT). The HTRF cAMP kit was purchased from Cisbio Bioassays (Bedford, MA).

*Cisbio homogenous time-resolved fluorescence (HTRF) cAMP dynamic 2 cell-based assay*

HEK293 cells stably expressing *Aa*DOP2 (HEK-*Aa*DOP2) or the human $D_1$ dopamine receptor (HEK-h$D_1$) were maintained and cryogenically frozen as previously described (Meyer et al., 2012). To prepare for pharmacological analysis, cells were thawed and re-suspended in assay buffer (HBSS, 20 mM HEPES, 0.1% Fatty acid free bovine serum albumin). To remove cryogenic freezing media, cell suspensions were centrifuged at 500xg for 5 min, followed by aspiration of the supernatant. Cell pellets were re-suspended in assay buffer and seeded into 384-well plates (Perkin Elmer CulturPlate-384) at 2,000-2,500 cells per well and incubated at

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37°C and 5% $CO_2$ for 1 h. Test compounds were added using a 384-well pin tool (V&P Scientific). A MultiFlo (BioTek) low-volume bulk reagent dispenser was used to dispense 3 μM dopamine (in assay buffer containing 500 μM IBMX and 0.02% ascorbic acid) to activate *Aa*DOP2. Drug stimulation was carried out at room temperature for 1 h. Cells were lysed by sequential addition of cAMP-d2 and anti-cAMP cryptate conjugate, both diluted 1:39 in lysis buffer and were incubated at room temperature for 1 h. Time-resolved fluorescence resonance energy transfer (TR-FRET) was measured with a lag time of 100 μs and integration time of 300 μs using a Synergy4 (BioTek) fluorescence plate reader with a 330/80 nm excitation filter and emission filters of 620/10 nm 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 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 above, but 500 nM dopamine was used to stimulate cAMP accumulation.

In vivo Aedes aegypti *larval screen.*

Test compounds were evaluated for *in vivo* toxicity in bioassays against L3 stage *A. aegypti* larvae in a double-blind manner. Briefly, compounds were re-suspended 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 Figure 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 h. Larvae unresponsive to gentle tapping of the plate or touch with a sterile probe were scored as dead.

Aedes aegypti *adult CRCs.*

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 per dilution of test compound (0.5 µl per mosquito) or *Aedes* saline alone (control) using a pulled glass capillary needle. Additional un-injected mosquito controls were also included. Mosquitoes were housed in 10 cm diameter x 20 cm height paper coffee cup cages with lace screen (secured with rubber bands) and maintained at 75% humidity with 10% sucrose provided *ad libitum* via a cotton wick (See Supplemental Figure 2 for illustrations of injections and mosquito housing). Observations of mortality were made daily for up to 4 d 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 individuals did not recover and died by assay end-point. 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 non-linear 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 pharmacological 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 384-well format, and initial experiments were focused on validating cAMP responses to dopamine stimulation using this assay format. As *Aa*DOP2 is a Gαs-coupled D1-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) (Hill et al., 2013; Meyer et al., 2012), demonstrating suitability of the HTRF cAMP detection technology for high-throughput cell-based pharmacological studies on *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, pharmacological evaluation of additional small molecules that are structurally related to the tricyclic leads was carried out in the cell-based assay described above. 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 (Figure 1 and Table 1).

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* SAR analysis. As performed above, 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 sub-nanomolar $IC_{50}$ values for inhibition of dopamine-stimulated cAMP in HEK-*Aa*DOP2 cells (Table 2).

Pharmacological 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-h$D_1$ cells) and compared to the h$D_1$ antagonist, SCH-23390. Each compound inhibited 500 nM-dopamine-stimulated cAMP in the HEK-h$D_1$ cells and displayed $IC_{50}$ values between 19 and 13000 nM (Table 3). However, in contrast to the h$D_1$-selective antagonist SCH-23390, all of these compounds were more potent antagonists of *Aa*DOP2 than h$D_1$, suggesting potential species-selective pharmacological profiles for these compounds.

In vivo *toxicity of* Aa*DOP2 antagonists: Effects on* Aedes 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 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 three hours (data not shown). We also identified five compounds with moderate mosquito toxicity (i.e., 40-70% mortality at 24 hours post exposure), 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 ($r = -0.770$, $n = 25$, $p > 0.0001$; Figure 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* Aedes aegypti

Toxicity to adult female *A. aegypti* is considered an important property of any lead molecule because adult female mosquitoes are the only developmental stage 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 (Figure 3 and 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 un-injected controls throughout the 96 h experiments. Cis-(*Z*)-flupenthixol was the most potent compound, having an $LD_{50}$ of 1.26 nmol/mosquito following 24 h exposure (Figure 3). Chlorpromazine and cis-(*Z*)-flupenthixol became more potent over the course of the four day assay, as $LD_{50}$ values decreased by ~2-3 fold from the 24 to 96 h 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 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, 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* (Figure 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 nM and 570±110 nM, respectively). Amperozide and benztropine interact with several different mammalian GPCR families (Arnt and Skarsfeldt, 1998; Bolden et al., 1992; Kanba and Richelson, 1984; U'Prichard et al., 1977), 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 physic-chemical 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 (Tables 1 and 2 and Supplemental Figure 3). However, the moderate potency of R59-022, risperidone, benztropine, and amperozide ($IC_{50}$ values ranging from 53-570 nM) indicate 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 (Figure 4A). Another SAR trend suggested that greater lipophilicity may enhance *Aa*DOP2 antagonist potency as was observed by the ~3-fold greater potency of amitriptyline

over doxepin (Figure 4B). Furthermore, ligands with tertiary amines (clomipramine, imipramine, amitriptyline, and loxapine) were ~5-100-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 (Figure 4C and Supplemental Figure 3). Also, clomipramine and norclomipramine displayed ~5-6 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 (Figure 4C). Enhancements in *Aa*DOP2 antagonist potency were also apparent when considering combinations of chemical properties such as lipophilicity and halide substitution (Figure 4C, clomipramine vs. desipramine) or lipophilicity and conformational rigidity (Figure 4D). The chemical scaffolds identified above and their key structural features that contribute to *Aa*DOP2 antagonism may be utilized 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 biological activity in *A. aegypti* but not in humans or other higher eukaryotes. To date, all reports of compounds that display both *Aa*DOP2 antagonism and *in vivo* efficacy are known to have biological 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 biological 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., 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.

Pharmacological selectivity considerations are multi-fold, as ligand selectivity for *Aa*DOP2 receptors over non-target insects (e.g., honeybees), in addition to selectivity over human GPCRs, is also paramount. Pharmacological screening panels can be assembled for invertebrate targets to better understand ligand pharmacology at these receptors. For example, cross-species comparative pharmacological studies of invertebrate dopamine receptor modulation can be expanded to include GPCRs from non-target 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 pharmacological 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* pharmacological evaluation and SAR profiling, while offering several advantages over the previously utilized CRE-mediated luciferase reporter assay for HTS. Direct measurement of cAMP eliminates false-positives associated with CRE-luciferase reporter assays that include cAMP/PKA-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 384-well format in singlet (i.e., Z' > 0.5, *unpublished observations*), enabling sufficient throughput to carry out the *in vitro* pharmacological profiling proposed above. The HTRF screening platform also provides flexibility, as it can be used to detect modulation of additional downstream GPCR signaling pathways including ERK1/2 and $Ca^{2+}/IP_3$ (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 above, 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 (Hill et al., 2013; Meyer et al., 2012). 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 above can be combined to expedite the discovery of novel ligands that selectively modulate GPCRs of target insects.

The present study describes the *in vitro* pharmacological characterization and *in vivo* efficacy of several *Aa*DOP2 antagonists and demonstrated 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 demonstrated 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, and Watts

Conducted experiments: Conley, Doyle, Meyer, and Nuss

Contributed new reagents or analytical tools: Hill and Watts

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

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

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**Footnotes**

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b) Address correspondence to:

Val J. Watts, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907, U.S.A. E-mail: wattsv@purdue.edu

**Figure Legends**

Figure 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 mean ± S.E.M. for at least three independent experiments.

Figure 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 h 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.

Figure 3. Concentration-response curves of adult *Aedes aegypti* female mortality 24 h after injection with *Aa*DOP2 antagonists. Each data point represents mean ± S.E.M. for three independent experiments. No mortality was observed in saline-injected or un-injected controls at the 24 h timepoint.

Figure 4. Structure-activity relationship trends for *Aa*DOP2 receptor antagonists. Compound names and *in vitro* $IC_{50}$ values (nM) for *Aa*DOP2 antagonism were included.

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**Tables**

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** | **$IC_{50}$±SEM (nM)** | **Chemical Class** |
|---|---|---|
| (+)-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 | D1DR antagonist |
| Trazodone | No Inhibition[a] | SARI |
| Venlafaxine | No Inhibition[a] | SNRI |

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

D1DR, selective D1-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

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Table 2. Pharmacological 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 | 18000±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 |

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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-h$D_1$ cells. The data represent the mean ± S.E.M. $IC_{50}$ values for four independent experiments.

| **Compound** | **$IC_{50}$±S.E.M. (nM)** | **Relative fold selectivity (*Aa*DOP2/h$D_1$)** |
|---|---|---|
| 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 |

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Table 4. *In vivo* toxicity of test compounds to *Aedes 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 |

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Table 5. Toxicity of injected *Aa*DOP2 antagonists to four-day-old adult female *A. aegypti*. $LD_{50}$ values (nmol/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 un-injected 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 |

Figure 1

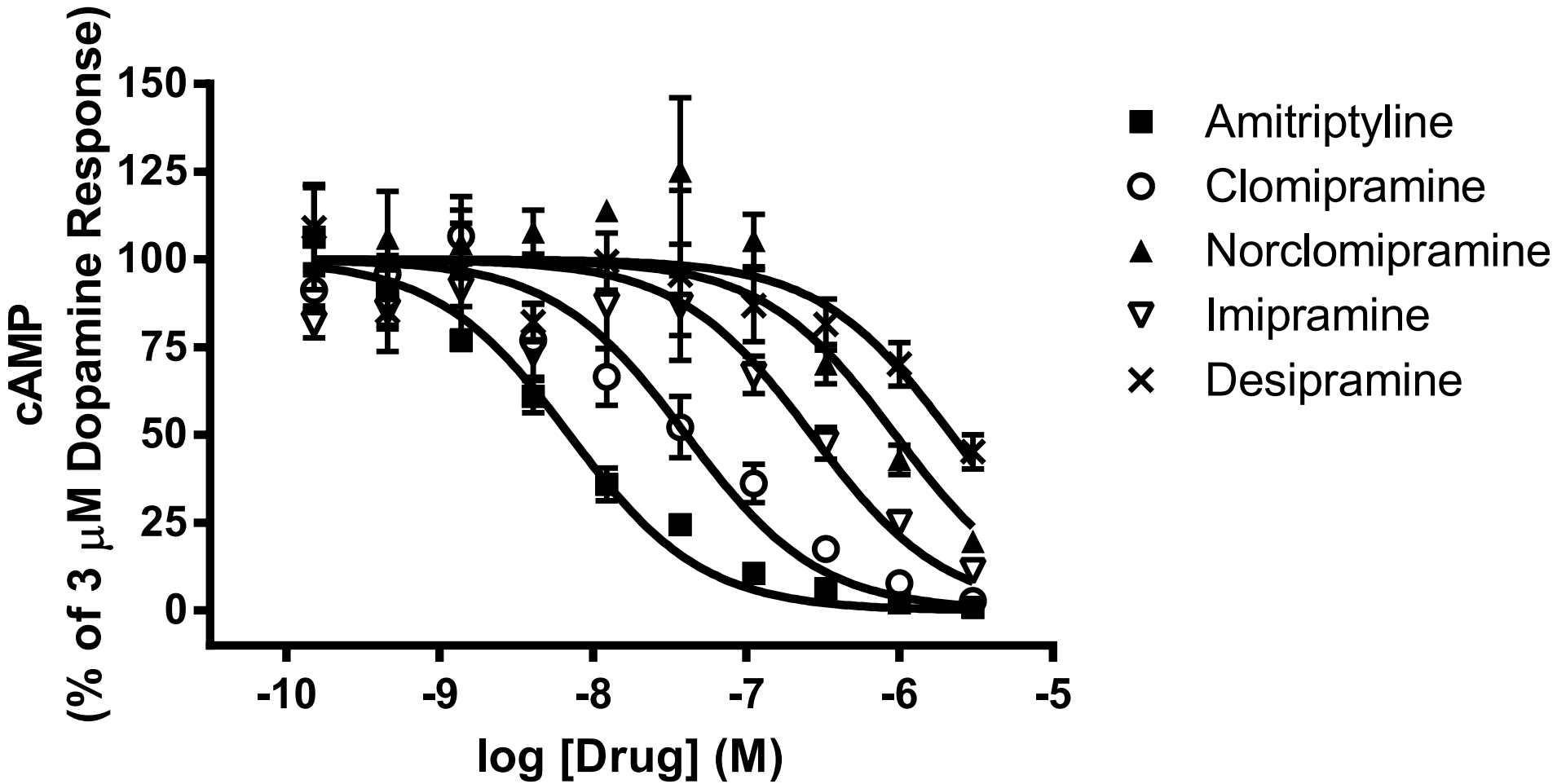

cAMP
(% of 3 μM Dopamine Response)
150
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
log [Drug] (M)
Amitriptyline
Clomipramine
Norclomipramine
Imipramine
Desipramine

Figure 2

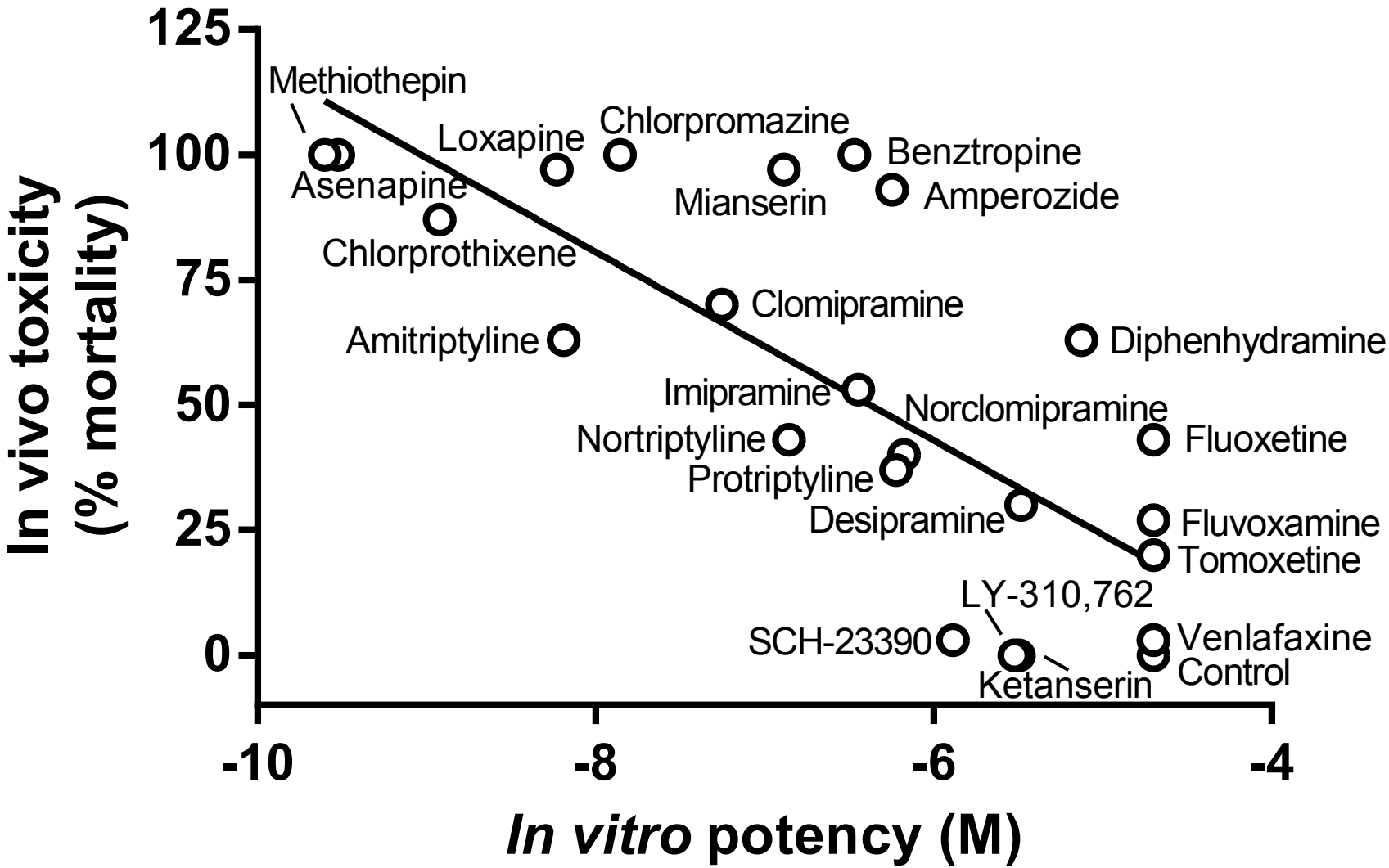

125
100
75
50
25
0
In vivo toxicity
(% mortality)
-10
-8
-6
-4
In vitro potency (M)
Methiothepin
Asenapine
Loxapine
Chlorpromazine
Benztropine
Mianserin
Amperozide
Chlorprothixene
Clomipramine
Amitriptyline
Diphenhydramine
Imipramine
Norclomipramine
Nortriptyline
Fluoxetine
Protriptyline
Desipramine
Fluvoxamine
Tomoxetine
LY-310,762
SCH-23390
Venlafaxine
Control
Ketanserin

**Figure 3**

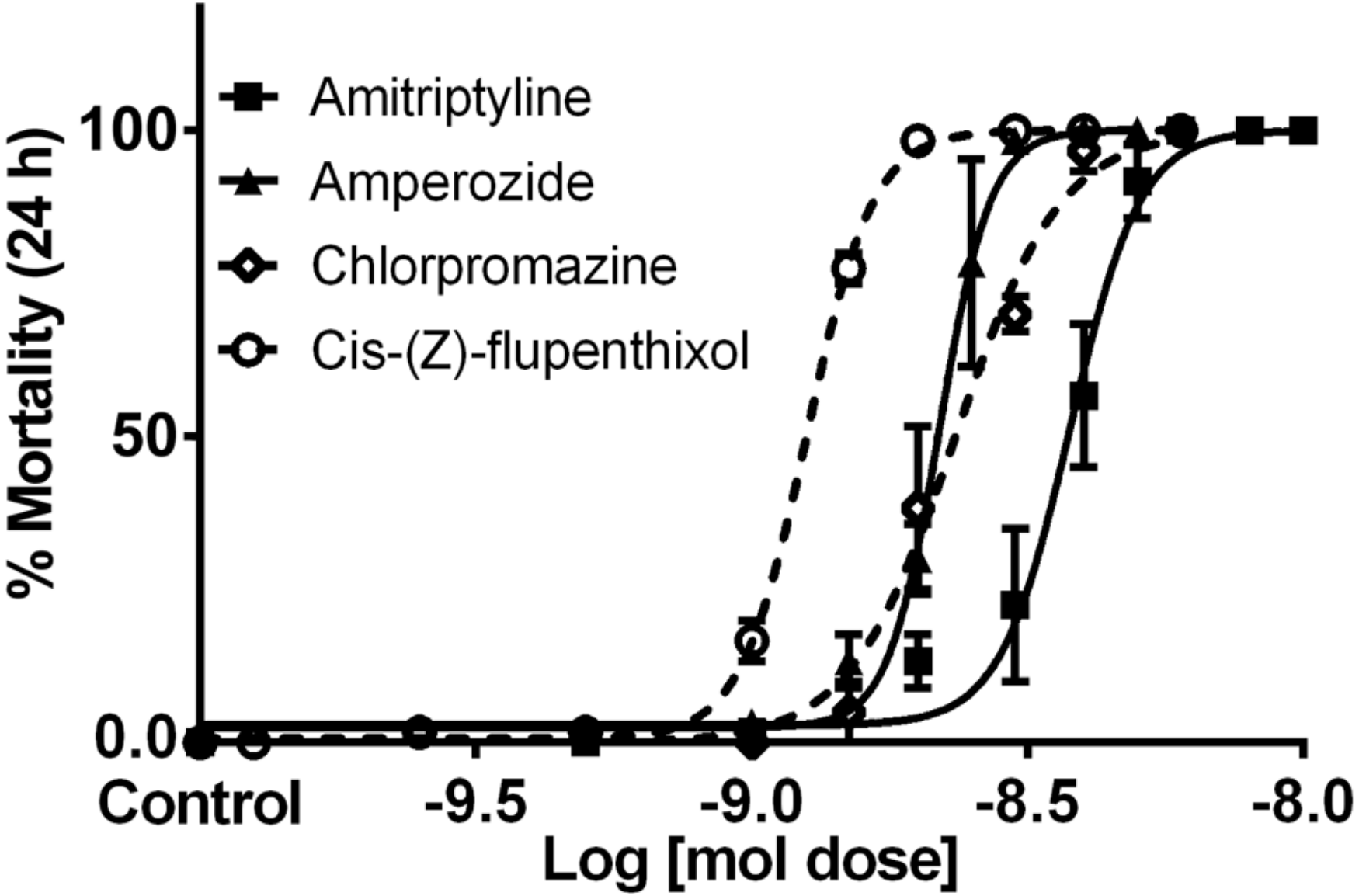
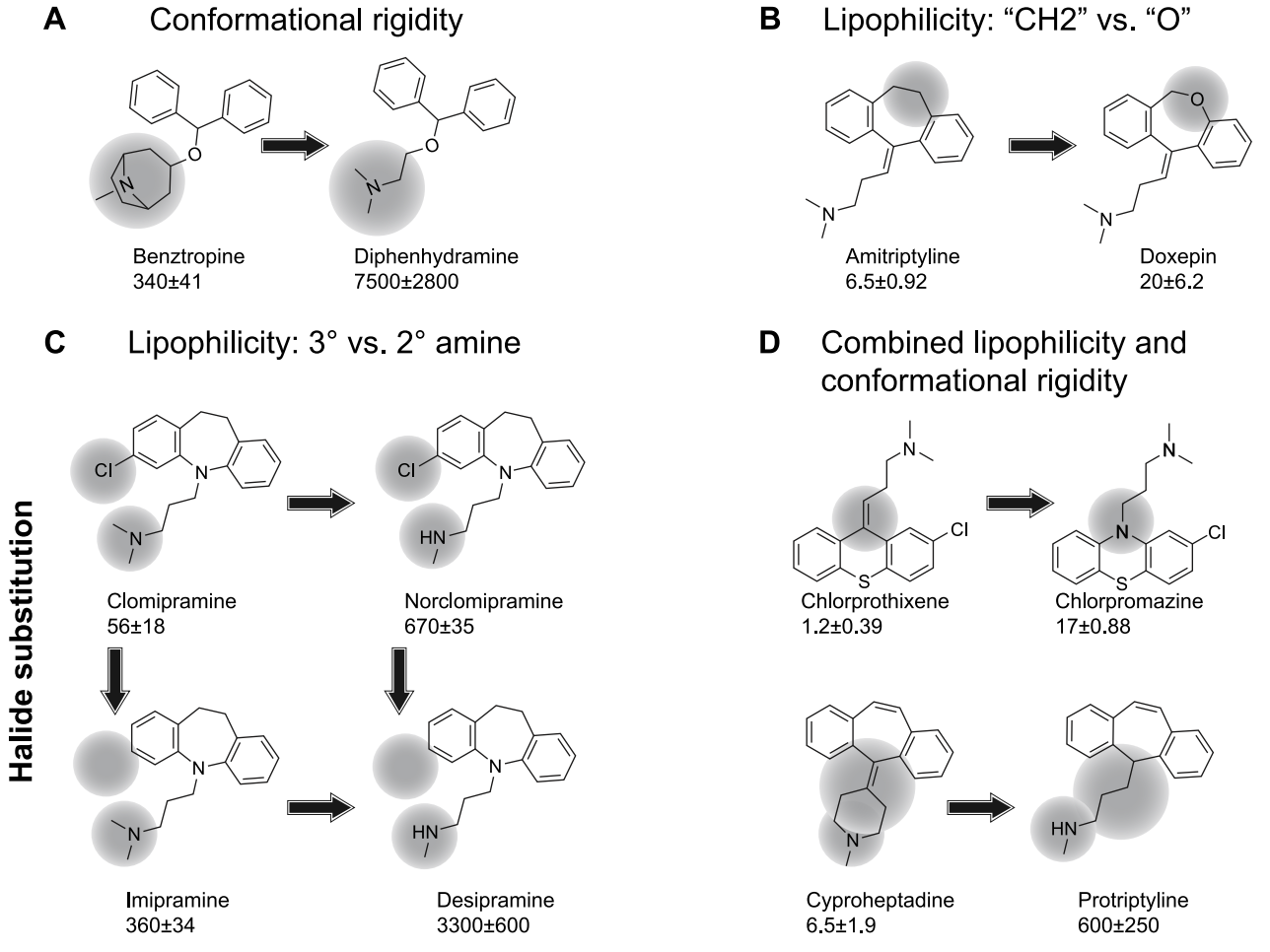

Amitriptyline
Amperozide
Chlorpromazine
Cis-(Z)-flupenthixol
% Mortality (24 h)
100
50
0.0
Control
-9.5
-9.0
-8.5
-8.0
Log [mol dose]

**Figure 4**


A
Conformational rigidity
Benztropine
340±41
Diphenhydramine
7500±2800
B
Lipophilicity: “CH2” vs. “O”
Amitriptyline
6.5±0.92
Doxepin
20±6.2
C
Lipophilicity: 3° vs. 2° amine
Halide substitution
Clomipramine
56±18
Norclomipramine
670±35
Imipramine
360±34
Desipramine
3300±600
D
Combined lipophilicity and conformational rigidity
Chlorprothixene
1.2±0.39
Chlorpromazine
17±0.88
Cyproheptadine
6.5±1.9
Protriptyline
600±250