HHS Public Access

Author manuscript

Physiol Entomol. Author manuscript; available in PMC 2020 April 03.

Published in final edited form as:

Physiol Entomol. 2012 March ; 37(1): 33–41. doi:10.1111/j.1365-3032.2011.00821.x.

Novel high-throughput screens of *Anopheles gambiae* odorant receptors reveal candidate behaviour-modifying chemicals for mosquitoes

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Abstract

Despite many decades of multilateral global efforts, a significant portion of the world population continues to be plagued with one or more mosquito-vectored diseases. These include malaria and filariasis as well as numerous arboviral-associated illnesses including Dengue and Yellow fevers. The dynamics of disease transmission by mosquitoes is complex, and involves both vector competence and vectorial capacity. One area of intensive effort is the study of chemosensory-driven behaviours in the malaria vector mosquito *Anopheles gambiae* Giles, the modulation of which are likely to provide opportunities for disease reduction. In this context recent studies have characterized a large divergent family of *An. gambiae* odorant receptors (AgORs) that play critical roles in olfactory signal transduction. This work has facilitated high-throughput, cell-based calcium mobilization screens of AgOR-expressing HEK cells that have identified a large number of conventional AgOR ligands, as well as the first non-conventional Orco (olfactory receptor co-receptor) family agonist. As such, ligand-mediated modulation serves as a proof-of-concept demonstration that AgORs represent viable targets for high-throughput screening and for the eventual development of behaviour-modifying olfactory compounds. Such attractants or repellents could foster malaria reduction programmes.

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Keywords

Active odorants; anopheline; control; host selection; mosquito; odour receptors; high-throughput screen

Introduction

A multitude of field and laboratory studies have linked female mosquito host preference to olfactory signals (Dekker *et al.*, 1998, 2001; Mboera *et al.*, 2000) and particularly human odours in the case of *An. gambiae* (For reviews see Bock & Cardew 1996; Takken & Knols, 1999 & 2009; Zwiebel & Takken, 2004; Besansky *et al.* 2004). Indeed, the propensity of female *Anopheles gambiae* mosquitoes to select humans for blood-feeding (anthropophily) is directly correlated to some of the highest-recorded Human Biting Indexes, (Tanga *et al.,* 2011). The strong anthropophily in this system is furthermore demonstrated in two-choice studies in an olfactometer where *An. gambiae* moves significantly more towards human odour than does a sibling species *An. quadriannulatus* (Pates *et al.*, 2001a). Moreover, *An. gambiae* shows a strong aversion to cow odour while *An. quadriannulatus* has no preference for either odour source (Pates *et al.*, 2001a). In other field trials, *An. gambiae* has demonstrated a strong preference for human hosts from a distance, even in the absence of visual cues or CO_2, while its sibling species *An. arabiensis* moves more towards animal odours (Costantini *et al.*, 1996, 1998). Field collections of mosquitoes using tent traps containing sleeping humans, again without the possibility of visual contact between the mosquito and host, also catch large numbers of female *An. gambiae* mosquitoes. Odour-releasing traps routinely catch more Anophelines when human odours are used (Njiru *et al.*, 2006), even when in direct competition with primate odours. Taken together these studies make a strong case for the use of novel olfactory-based interventions to reduce human-vector encounters.

The location of a blood-meal source involves a series of behaviours: activation of insect flight following stimulation with a host chemical odour (kairomone), upwind flight in the direction of the odour, and eventually alighting/probing on the host itself. In the context of human odour, approximately 350 different chemical compounds have been identified in sweat (Cork & Park, 1996). Attractive sweat compounds include carboxylic acids (Meijerink, 2001; Meijerink & van Loon, 1999), ammonia (Meijerink, 2001), lactic acid, and various other volatiles (Cork & Park, 1996; Meijerink *et al.*, 2001; Healy & Copland 2001). Moreover, *An. gambiae* is more responsive to incubated human sweat than freshly-collected sweat (Braks *et al.*, 2001). Skin microbes are responsible for the changes in chemical composition of sweat that increase its potency (Verhulst *et al.*, 2009;). *An. gambiae* females also respond to human breath components, although though this response is sometimes based on repellent effects (Mukabana *et al.*, 2004; Qiu *et al.*, 2010). Another important component of human breath is CO_2. While not a human-specific odour, CO_2 has long been recognized as an important mosquito kairomone in both field and laboratory settings conditions (Costantini *et al.*, 1996). Carbon dioxide acts synergistically to enhance the response to other volatiles and is particularly important for the activation phase of host seeking (Takken and Knols, 1999; Dekker *et al.*, 2001; 2005; Lacey & Cardé, 2010). As is

the case for CO_2, ammonia also acts as a powerful synergist for human hosts (Smallegange *et al.*, 2005).

The molecular basis of *Anopheline* chemosensation

At the molecular level, insect olfactory signal transduction results from the interaction of chemical odorants and several groups of proteins expressed on the peripheral dendrites of olfactory receptor neurones (ORNs) that typically are found on the antennae, maxillary palps and other adult head appendages. These include odorant binding proteins (OBPs), odorant degrading enzymes (ODEs), sensory neuron membrane proteins (SNMPs) as well as large families of variant ionotropic and odorant receptors (IRs and ORs, respectively) that have been extensively reviewed elsewhere (Rutzler & Zwiebel, 2005; Benton, 2009).

Because of their central role in olfactory signal transduction OR proteins have been the subject of extensive study. The first insect odorant receptors (*Ors*) were molecularly identified in *Drosophila melanogaster* by multiple groups using differing approaches (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999). As a result of genome sequencing projects, *Or* gene families have now been described in numerous insect species representing multiple orders (Krieger *et al.*, 2003; Robertson *et al.*, 2003; Robertson and Wanner, 2006; Smadja *et al.*, 2009; Smith *et al.*, 2011), including three mosquito species: *An. gambiae* (Hill *et al.*, 2002), *Ae. aegypti* (Bohbot *et al.*, 2007), and *Culex quinquefasciatus* (Pelletier *et al.*, 2010). With one notable exception (see below) insect *Ors* are an extremely divergent gene superfamily, often sharing very low amino acid identities/similarities within the same species, and having few orthologs between species (Robertson *et al.*, 2003; Ache & Young, 2005; Robertson, 2006).

The large family of 79 candidate *AgOr*s was identified using homology-based approaches (Fox *et al.*, 2001, 2002; Hill *et al.,* 2002). Of these 79, 75 appear to be transcriptionally active (Pitts *et al.*, 2011). As is the case for other insect *Ors*, the majority of *AgOr* gene products share little identity at the primary amino acid level, usually less than 20%, although 14 pairs share >70% identity (Hill *et al.*, 2002). In the olfactory tissues of insects, individual ORNs appear to express only one of these conventional ORs (also called “tuning” ORs), and it is members of this class of *Or* that then defines the unique odorant response profile of any given ORN (Wetzel *et al.*, 2001; Sakurai *et al.*, 2004; Neuhaus *et al.*, 2005).

In stark contrast to the diversity and expression profiles of conventional, tuning *Or*s, one member of this gene family which is now uniformly known as *Orco* (olfactory receptor co-receptor) is both highly conserved across insect orders and is ubiquitously expressed in all ORNs. *Orco* is necessary and sufficient for the proper localization and retention of other conventional O*rs* at the dendritic membrane (Larsson *et al.*, 2004, Benton *et al.*, 2006), and is required for proper function of conventional *Ors* (Krieger *et al.*, 2003; Pitts *et al.*, 2004; Larsson *et al.*, 2004; Jones *et al.*, 2005; Xia & Zwiebel, 2006). This exceptional degree of sequence conservation and expression characteristics among insect OR gene families suggested that the *Orco* subfamily represents a non-conventional *Or* that is broadly required for olfactory signal transduction in all insects.

Tuning *Ors* from multiple insect species have now been functionally characterized and greater than 60% of AgORs have been functionally characterized through various heterologous expression methods (Lu *et al.*, 2007; Xia *et al.*, 2008; Carey *et al.*, 2010; Wang *et al.*, 2010). In each of these systems a tuning OR is expressed along with Orco; signal is not observed in the absence of Orco. Although AgOrs expressed in heterologous systems are generally not as sensitive as the native neurones in their ability to detect nanomolar odorant concentrations, they nevertheless faithfully recapitulate the agonist rankings of AgOR-expressing neurones (Lu *et al.*, 2007; Carey *et al.*, 2010, Bohbot *et al.*, 2011). This observation serves as a proof-of-concept that these expression systems are excellent substitutes for the native system, and provides a necessary rationale for extensive AgOR functional characterization in high throughput systems outside of the constraints of the mosquito.

Importantly, functional analyses using heterologous expression studies have revealed that on its own, *Orco* does not confer odorant sensitivity (Dobritsa *et al.*, 2003; Elmore *et. al.,* 2003; Benton *et al.*, 2006; Sato *et al.*, 2008; Wicher *et al.*, 2008). Rather, *Orco* forms an essential part of a multimeric ion channel in cooperation with a tuning *Or* that is gated by its cognate odour ligand (Sato *et al.*, 2008; Wicher *et al.*, 2008; Smart *et al.*, 2008) and elicits intracellular signals without GTP-binding proteins (Sato *et al.* 2009, Jones *et al.*, 2011). Validation of these paradigms *in vivo* remains an important objective of current efforts.

Molecular targets of current insect repellents

The molecular mechanisms and targets of known insect repellents have been the subject of considerable study. While still somewhat controversial, several studies suggest that the most effective and wide used commercial repellent, N,N-Diethyl-meta-toluamide (DEET) triggers an aversive response through the bimodal activation of a subset of ORNs and specific Gustatory Receptor Neurones (GRNs; Syed & Leal, 2008; Xia *et al.*, 2008; Liu *et al.*, 2010). Citronella, the only other insect repellent with at least a partially-defined molecular target, activates a Transient Receptor Potential (TRPA1) channel that is normally responsible for heat detection in dipterans to trigger an avoidance response (Kwon *et al.*, 2010). In addition, citronella also targets an Orco dependent pathway, although the conventional OR targets have yet to be defined (Kwon *et al.*, 2010). However, despite its widespread use, DEET is somewhat toxic and hardly affects some vector mosquito species; factors that decrease consumer acceptance (reviewed in Paluch *et al.*, 2010). For these reasons, there is widespread interest in developing next generation insect repellents, with increased efficacy, longer half-lives, and lower toxicity.

High throughput approach to insect control by modifying behaviours

Until recently, the development of new attractants and repellents for insects has largely focused on screening compounds using electrophysiology and whole-organism behavioural responses. Such approaches are resource-intensive, require large amounts of compound, and are not amenable to high-throughput approaches (Paluch, *et al.*, 2010). In an effort to circumvent this bottleneck, the feasibility of employing high throughput, cell-screening technologies that have been principally focused upon pharmacologic drug discovery has been investigated. A critical component of such an undertaking is the ability to faithfully

express screening targets in one or more heterologous systems in order to facilitate rapid and sensitive screens of small molecule libraries. As AgORs are key mediators of various mosquito behaviours and have been shown to express well in various heterologous systems, AgORs have been selected to be expressed in HEK293 cells (always in conjunction with the co-receptor, AgOrco) for the purposes of high throughput screening. These screens are designed to identify novel AgOR modulators that could potentially affect Anopheline behaviour (Lu *et al.*, 2007; Carey *et al.*, 2010, Wang *et al.*, 2010; Bohbot *et al.*, 2011).

In selecting the AgOR targets of these screens, specific AgORs are chosen from among the nearly 60 tuning ORs expressed in the antennal tissues of adult *An. gambiae*. Among these receptors, AgOR10 is seen as the prime candidate for extensive screening for two principal reasons. First, AgOR10 is one of only two AgORs that shows a greater than 69% conservation of peptide sequence between the blood-feeding mosquito subfamilies of *Anophelinae* and *Culicinae* (Bohbot *et al.*, 2011; LJZ unpublished data). Secondly, when expressed heterologously, AgOR10 demonstrates a robust responsiveness to known, attractive semiochemicals, thus suggesting that AgOR10 plays an active role in olfactory-mediated behaviours of hematophagous mosquitoes (Carey *et al.*, 2010, Wang *et al.*, 2010; Bohbot *et al.*, 2011). Consequently, a high-throughput screening protocol has been optimized around AgOrco + AgOR10 expressing HEK293 cells.

Materials and methods

Calcium fluorometry

The creation and validation of the AgOrco +AgOR10 cell line used in these screens has been previously described (Bohbot *et al.*, 2011). All assays were conducted using the instrumentation available through the high-throughput screening Facility that is part of the Institute for Chemical Biology at Vanderbilt University. Twenty four hours prior to each assay, cells were plated at a concentration of 20000 cells/well in a black-walled, clear-bottomed 384-well plate (Greiner Bio-One, Longwood, Florida) and incubated for 8 h at 37 °C with 5% CO_2 to facilitate adherence. Cells were then treated with 0.3 μg μL^{-1} tetracycline (Sigma-Aldrich) overnight to induce expression of the AgOR complexes. 12–16 h post-induction, cells were incubated for 45 min at 37 °C with 20 μL of 3 μM Fluo-4/acetoxymethyl ester (Fluo-4AM), prepared as a 2.3 mM stock in DMSO (Sigma-Aldrich), mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127 (Invitrogen), and diluted in assay buffer (Hanks' balanced salt solution, 20 mM HEPES, and 2.5 mM probenecid). Dye-buffer was then replaced with 20 μL of calcium assay buffer.

Vanderbilt Molecular Screening Library test compounds were prepared from 10 mM DMSO stocks, and diluted in assay buffer to 20 μM (i.e., 2× 10 μM) in the central 320 wells of a 384-well, 15mm poly-propylene plates using an Echo 555 acoustic liquid handler (Labcyte, Sunnyvale, California) and a Multidrop Combi (Themo Scientific). For the AgOR10 odour-control plates, EC_{20}, EC_{80}, and EC_{100} concentrations of 2-ethylphenol (Sigma Aldrich) were experimentally determined by half log-molar concentration response curves generated in the assay format employed. The odour-control compound was prepared for assay in each of either of two separate 384-well, deep well (22 mm) poly-propylene plates (Greiner Bio-One, Longwood, Florida). The first control plate contains a 5× EC_{20} (5 μM) concentration of 2-

ethyl phenol (2-EP) in each of the 356 central wells (i.e., all wells exclusive of the outer columns on east/west edges). The wells of the east/west edge outer columns then received either a 5× EC_{100} concentration of 2-EP (500 μM) or a DMSO-only negative control (EC_0). The second control plate contains a 5× EC_{80} (50 μM) concentration of 2-EP in each of the 356 central wells and assay buffer only in the east/west edge outer columns.

Fluorescence readings and integrated liquid handling were conducted in a Hamamatsu FDSS6000 plate reader (Excitation: 470 ± 20 nm; Emission: 540 ± 30 nm; Hamamatsu Corporation, Bridgewater, New Jersey). Each cell plate was assayed over the course of five min, and was subjected to of three separate compound additions interspersed with latency periods to allow for a return to baseline (Fig. 1). Compound additions were performed at assay time-points of 5, 120 and 220 s, from the test compound plate, the first control plate and the second control plate respectively. The volumes of each addition were 2l, 10, and 12 μL from the test compound plate (2× final concentration), the first control plate (5× final concentration) and second control plate (5× final concentration), respectively. Fluorescent readings were taken at 1-s intervals over the course of the entire assay. Additional, 0.5-s fluorescent readings were taken during the 30 s subsequent to each addition to better quantify response kinetics.

Hit identification

The following criteria and related algorithm were used to automatically identify (call) hits. Agonists were called if and only if: **[A1+x]>(1.05*[avgEC_0+x]) AND [A3+x]<[EC_{80}+x]** Where [A1+x] is the RFU (Relative Fluorescent Unit) taken at x sampling intervals after the first compound addition (A1; Fig. 1). The agonist criteria requires both a fluorescent increase of 5% over background (RFU of the avgEC_0 at time x) to demonstrate efficacy, as well as a cumulative, time-dependent diminution in RFU following the third addition (A3). This latter criteria was employed to filter out both auto-fluorescing compounds as well as compounds that may have off-target effects

Potentiators (compounds that have no intrinsic activity, but that can amplify the activity of other agonists) were called if and only if: **[A2+x]−[A2−3]>(1.2*[avgEC20+x]−[avgEC20-3])**. Criteria for a potentiator required an RFU increase of greater than 20% (over control) in wells receiving an EC_{20} concentration of agonist control.

Antagonists were called if and only if **[A3+x]<(0.7*[EC80+x]) AND [A1+x]=[A1+x+10]** where A3 represents the third add, containing an EC_{80} concentration of control compound. The antagonist criteria requires that a given test compound be able to diminish the signal of an EC_{80} concentration of control compound by 30-percent or more. Additionally, it was that the test compound itself have no intrinsic efficacy.

Hit confirmation

To confirm potentially-active small-molecules identified in the primary screen, cells were plated and assayed as they were in the primary screen. Furthermore, to discriminate OR agonists from compounds capable of generating non-specific responses from HEK cells, all agonists were tested against uninduced HEK cells. Compounds were confirmed in three or

more replicate assays in both tetracycline-induced cells and in uninduced cells. Only those small molecules that showed activity in the induced line and no activity in the uninduced line were classified as “confirmed hits”.

Results and Discussion

Z-factor determination

To determine if the resolution of this assay was suitable for high-throughput screening, the Z-factor was calculated within pilot experiments (Zhang et.al.,1999). A Z-factor was determined between the EC_0 (agonist) and EC_{80} (antagonist) window. A Z-factor of 0.79 was found for the EC_{80} positive control (Figure 2), confirming that this assay has powerful hit discrimination capabilities

Screen design

The high throughput screening project was designed to identify novel modulators of mosquito ORs, which could potentially translate to behavioural-control agents for disease reduction programmes. AgOR10 was chosen both for its high signal levels in the presence of behaviourally-associated odorant stimuli and for its demonstrated functional conservation across mosquito species. In these assays, AgOR10 was co-expressed with AgOrco in HEK cells under a tetracycline inducible system and was validated as described (Bohbot *et al.*, 2011).

The format of the screen was designed as a “triple-add” assay, which takes maximal advantage of the automated liquid handling and plate-reading capabilities of the FDSS instrumentation to examine a given compound from the small-molecule library for intrinsic, AgOR10 agonism or its ability to potentiate or antagonize the receptor’s response to a positive control in a single well over a 5-min time course visualized in real-time (Fig. 1). This was an efficient method to identify as many modulators as possible without losing resolution. In the first add, library compound was added to the experimental well to a final concentration of 10 uM and the library compound’s agonist capacity was examined. Subsequently, in the second add, an [EC_{20}] of 2-EP was added to each well to determine the compounds’ ability to potentiate the ORs response to cognate ligand. Lastly, in the third add, an [EC_{80}] of 2-EP was added to each test well to test for antagonism.

Odorant-based variation in the context of high-throughput screening

Initial pilot experiments involving various AgORs and their associated, odorants, revealed several phenomena that seemed to directly relate to the intrinsic volatility of the low-molecular weight odorants being employed as positive controls. Of particular note, it was observed that control plates containing the EC_{20} and EC_{80} concentrations of some odorants gradually lose their ability to elicit an equally efficacious response across successive cell plates, and that this decline in observed efficacy was odour-dependent. While some odours displayed remarkably stable efficacies across multiple, successive cell plates, the efficacy of other odours showed a marked, time-dependent decrease, even over the course of 2 or 3 plates. In the case of AgOR10, the 2-EP odour control displayed stable efficacies across multiple cell plates and allowed for very time-efficient screening. Conversely, in the case of

more-volatile odour controls, it was necessary to replace the control plates much more frequently. In addition to the odorant-dependent phenomena of decreased efficacy, some highly-volatile odorants also displayed a tendency to "bleed over" into neighbouring wells such buffer-only control additions positioned adjacent to EC_{100} control wells could sometimes elicit cell responses. Numerous tests were conducted to rule out the possibility of technically-induced odour contamination and to rule out possibility that high signal levels in the EC_{100} control wells was causing spurious readings in the adjacent wells. This phenomenon has been observed for a number of cell lines and odorants and disappears when ligands of higher molecular weight are included. AgOR10 and 2-EP did not display this bleed-over effect.

Agonist hit identification and analysis

Agonists were determined by an algorithm (defined in methods), which identified compounds with a response that was significantly greater than the baseline control and that also removed auto-fluorescent compounds. Two hundred and sixty-six agonists were identified in the initial primary screen. All hits were retested in duplicate against AgOR10+AgOrco cells as well as against un-induced cells to test for responses specific to the OR complex (Fig. 3). Revalidation screening confirmed that 68 of the original 266 agonists (~25%) maintained activity that was contingent upon AgOR10 expression. This is in keeping with typical high throughput screening efforts and most likely reflects errors in compound loading and/or identification.

AgOR10 tuning curves demonstrate that AgOR10 is strongly activated by a series of substituted aromatics (Bohbot *et al.*, 2011). The high-throughput screening agonist hit results were no different, as AgOR10 demonstrated strong responses to a number of complex aromatic compounds. Eight tractable hit scaffolds were identified which accounted for more than 70% of all confirmed hits. One of the largest hit series centred upon an imine moiety that encompassed 43 compounds. The true activity of these imine hits will need further verification as this class of compound is generally unstable in solution; acid hydrolysis of imines generally leads to the production of aldehyde and aniline degradation products, classes of compounds that may have intrinsic activity of their own. It is therefore possible that the degradation products of this one particular hit series would yield a diverse set of compounds that could only be further investigated using mass spectrometry analysis to confirm hit identity.

Potentiator and antagonist hit identification and analysis

There were a smaller number of identified potentiators, which had the capacity to increase the response of the control ligand, 2-EP in the second assay window. To be classified as a potentiator, a library compound needed to increase the response of the EC_{20} control by greater than 20%. Overall, 4 small molecules were identified and confirmed as potentiators of 2-EP in revalidation experiments. Surprisingly, no compounds were found that were capable of significantly inhibiting 2-EP-mediated activation.

A novel allosteric agonist

As an unintended consequence of the high-throughput screen for agents that could modify the activity of conventional mosquito AgORs, the first Orco agonist was discovered. This compound 2-(4-ethyl-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-ylthio)-N-(4-ethylphenyl)acetamide was originally thought to be an allosteric agonist of AgOR10 and was designated as such (VUAA1, Vanderbilt University Allosteric Agonist 1; Jones *et al.*, 2011). Indeed, of the more than 150000 compounds that have been screened against AgOR10+AgOrco from the Vanderbilt Small Molecule Library, VUAA1 is the only compound that displayed the hallmarks of allosteric agonism in that it possesses intrinsic agonism and it potentiates the response of 2-EP (Fig. 4). Interestingly, follow-up assays have demonstrated that VUAA1 activated multiple AgOR expressing cell lines, including AgOR8, AgOR10, and AgOR65 (L.J. Zwiebel, unpublished observation). As all of these cell lines also express AgOrco, it was possible that VUAA1 was acting directly on AgOrco. To test this hypothesis, cells expressing AgOrco and other Orco family members alone were treated with VUAA1; these cells display robust responses thereby confirming this molecule as the first-in-class ligand capable of directly gating AgOrco and other insect Orco ion channels (Jones *et al.*, 2011). In addition, these experiments also demonstrated that Orco can form stand-alone ion channels which are themselves capable of being activated directly. Furthermore, VUAA1 provides a powerful positive control that allows for a better examination of the results of previous studies which suggest that Orco is also a cyclic nucleotide gated channel (Sato *et al.*, 2008; Wicher *et al.*, 2008; Smart *et al.*, 2008); no evidence is observed for cyclic nucleotide gating of Orco. This analysis further redefines the model for insect odorant receptor signal transduction.

Conclusions

A calcium mobility assay that expresses and targets mosquito ORs has been developed in order to facilitate a novel high throughput screening-based approach to identify novel modulators of *Anopheline* ORs. Because advantage is taken of the pre-existing Institute for Chemical Biology at Vanderbilt University chemical library this initial effort has been biased towards molecules exhibiting known drug-like properties. These compounds are generally of a higher molecular weight than those chemical odorants thought to elicit responses from ORs in natural settings (Wang *et al.*, 2010; Carey *et al.*, 2010). These high molecular weights are generally indicative of low volatility, and thus the hits generated by these high throughput screening efforts are unlikely to be considered as a classical “odorant”. Therefore, lead characterization will likely involve further structure-activity relationship efforts to deconstruct these hits into lower molecular weight structures to increase volatility while maintaining efficacy as well as develop precise formulations to achieve this end. The identification and characterization of VUAA1 as an Orco family agonist raises the possibility of engineering broadly applicable formulations around this activity to hyper-stimulate the olfactory systems of most, if not all, insects. Efforts are currently engaged with behavioural validations of VUAA1 and other HTS hits against pre-adult and adult stage *An. gambiae*.

The ultimate aim of this work is to utilize state of the art, high-throughput screening-based approaches to develop a rapid method to begin to identify next generation insect excito-

repellents that could be used for a number of applications including agriculture, personal protection and public health. While there is, as yet, no comprehensive study that examines the feasibility of a repellent-based disease reduction programme, there is reason to posit that because known repellents have demonstrated remarkable protection against mosquito bites (approximately 96% in numerous field trials), an efficacious repellent would reduce disease burdens (Moore *et al.*, 2002; Costantini *et al.*, 2004). The few studies that have been performed suggest that with high compliance and repellent efficacy, malaria levels would drop below those achieved with insecticide treated nets (Moore, *et al.*, 2007). However, such studies have drawn on the benchmark mosquito repellent DEET, which has a number of limitations. In addition, in areas of high compliance, those users who do not apply repellent are put at greater risk because mosquitoes are diverted to non-compliant individuals; this raises a number of ethical problems (Moore *et al.*, 2007). The use of high-throughput screening-based approaches described here represents a unique opportunity to revisit the search for next-generation insect excito-repellents that may be used for multiple purposes against a wide range of economically and medically important insects.

Acknowledgments

The authors wish to thank staff members at The Vanderbilt Institute of Chemical Biology High-throughput Screening Facility for instrumentation support and members of the Zwiebel laboratory for helpful discussions. This work was supported by Vanderbilt University and a grant from the Foundation for the National Institutes of Health to L.J. Z through the Grand Challenges in Global Health Initiative.

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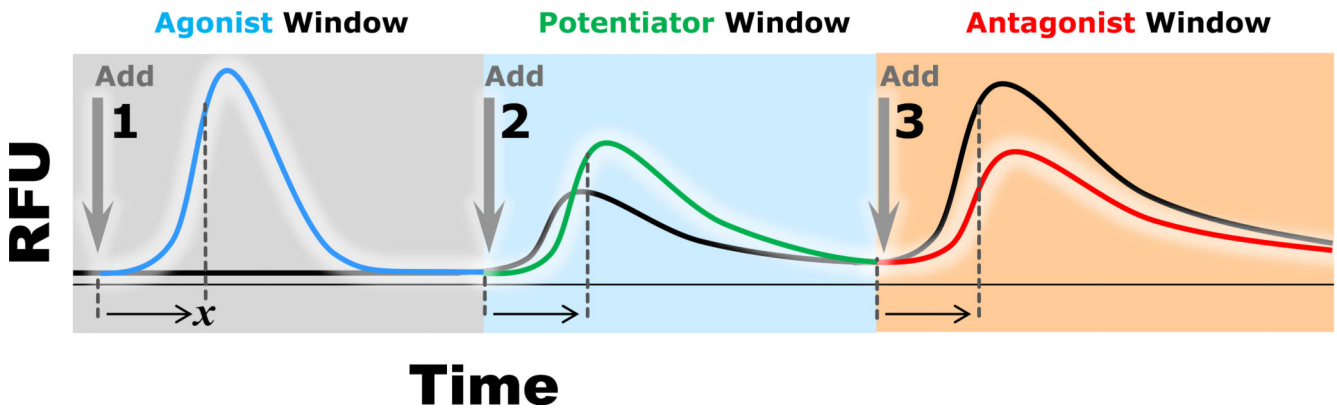

Figure 1.

Triple-add screen design. Well-by-well relative fluorescence units (RFU) are measured over a time course of 5.5 min. Compound activity is gauged against the RFU produced by control wells (black line). The first addition (Add 1) contains a 10 μM concentration of test compound and no control compound. The second addition (Add 2) is of an EC_{20} concentration of control compound and the third addition (Add 3) is one of an EC_{80} concentration of control compound. Agonist activity of a given test compound (blue line) is ascertainable following Add 1; potentiator activity of the compound (green line) is ascertainable following Add 2; antagonist activity of the compound (red line) is ascertainable following Add 3. Hit discrimination is algorithmically determined by comparing RFU intensities in each window following a set interval of time (***x***).

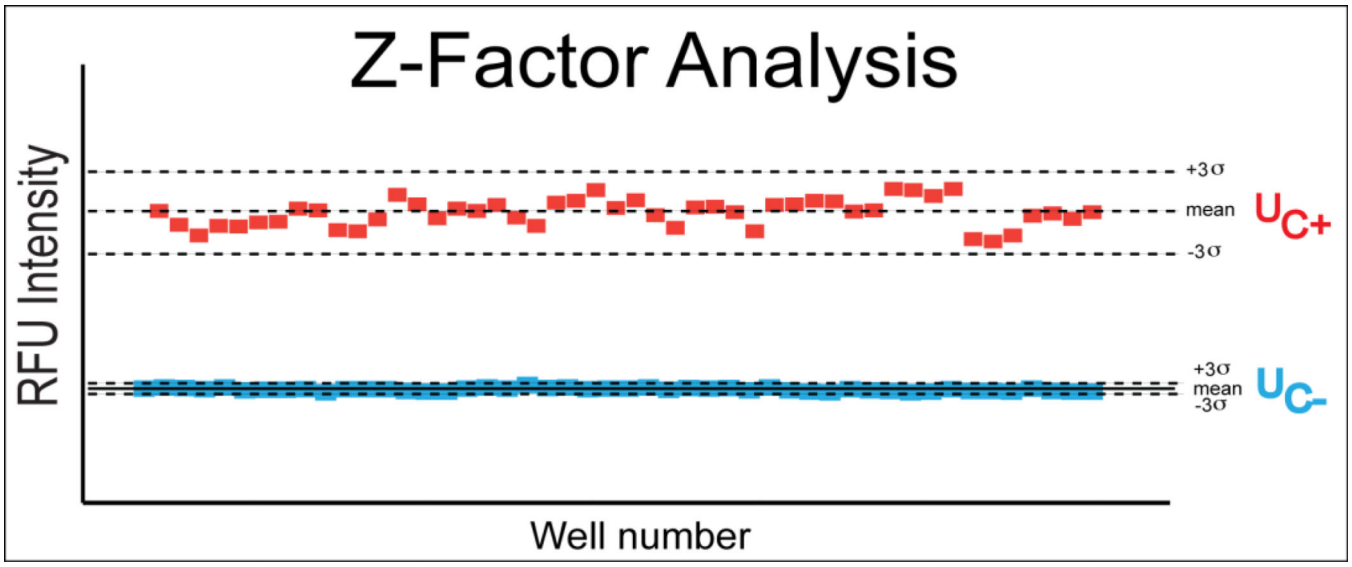

Figure 2.

Z- factor analysis of pilot experiments for EC_{80} concentrations of 2-ethyphenol and DMSO only control. Each data point represents the response to 2-EP (U_{c+}) or DMSO only (U_{c-}). Central dashed line for each group represents the mean, while upper and lower dashed lines represent (+/−) 3 standard deviations from the mean, respectively.

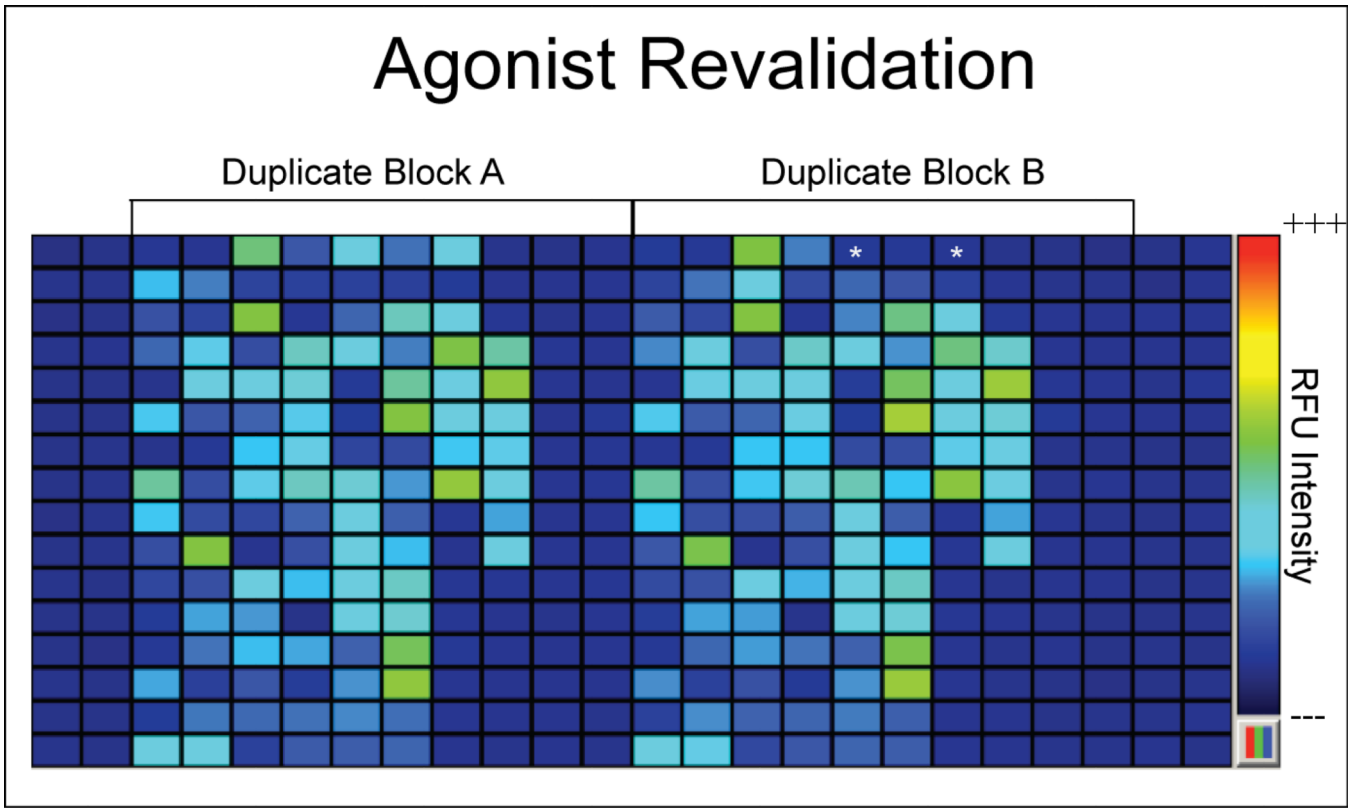

Figure 3.

A stylized schematic of a 384-well plate and the relative fluorescence unit (RFU) intensity for a representative agonist hit revalidation. Candidate hits were revalidated in duplicate blocks on the same 384-well plate to control for regional differences in RFU intensity scale as indicated using pseudo-colorization heat map from high (+++) to low (---) as depicted on extreme right. (*) indicates an example where a hit revalidated in one block, but failed to revalidate in another block.

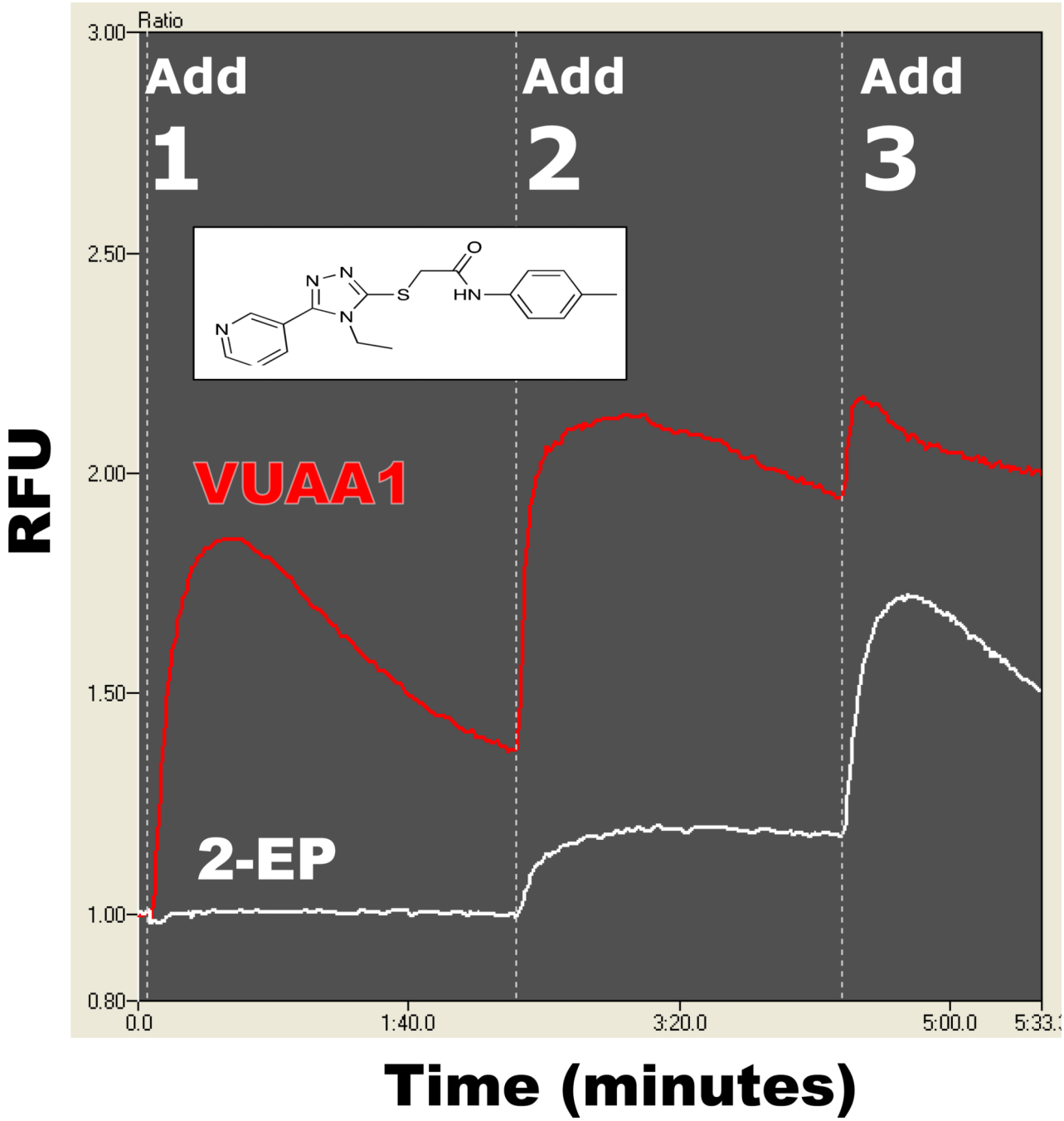

Figure 4.

Identification of a novel allosteric agonist. Activity of a novel compound is shown (red) and is compared to the control-only response (white). The compound (VUAA1, structure provided in inset) demonstrates the unique characteristics of both a stand-alone agonist and strong potentiating activator (Jones *et al.*, 2011).