Odorant receptor modulation: Ternary paradigm for mode of action of insect repellents

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1. Introduction

Perception of chemicals in the environment by insects begins when compounds activate ionotropic receptors, gustatory receptors and odorant receptors (ORs) located on the dendritic surface of chemosensory neurons (Kaupp, 2010). The latter receptor clade belongs to a rapidly evolving insect-specific gene family (Spehr and Munger, 2009) encoding membrane receptors of unknown subunit stoichiometry (Sato et al., 2008) and for which the ligand binding sites remain to be identified. Despite their sequence diversity, all ORs appear to share common structural and functional properties: (1) putative seven transmembrane-spanning domains (Mombaerts, 1999); (2) intracellular N-terminus and extracellular C-terminus (Benton et al., 2006; Lundin et al., 2007; Smart et al., 2008; Tsitoura et al., 2010); (3) receptor-co-receptor (ORx-Orco) complex formation (Benton et al., 2006; Neuhaus et al., 2005); and (4) ligand-binding capabilities (Hallem et al., 2004). A functional OR subtype is believed to be formed by the assembly of an obligatory OR co-receptor (Orco) acting as ion-channel (Jones et al., 2011; Nichols et al., 2011; Wicher et al., 2008), and a variable ligand-selective subunit (Carey et al., 2010; Hallem et al., 2004; Hallem and Carlson, 2006; Wang et al., 2010) gated by a broad spectrum of extracellular ligands (odorants). This macromolecular arrangement increases the likelihood of multiple recognition sites (orthosteric and allosteric), conferring behavioral states and complex interactions.

Evidence for these recognition sites is exclusively inferential as no three-dimensional structures of insect ORs have been determined to date. For this matter, mosquito ORs represent one of the most relevant systems to study new pharmacological compounds as well as their effects on behavior. For instance, indole, skatole and octenol are mosquito attractants produced by plants and animal waste products linked to feeding and oviposition (Du and Millar, 1999; Kline et al., 2007; Lindh et al., 2008; Meijerink et al., 2001; Meijerink et al., 2000; Millar et al., 1992; Takken et al., 2001). These compounds activate specific olfactory sensory neurons (OSNs) in the antenna and maxillary palp of mosquitoes (Blackwell and Johnson, 2000; Cook et al., 2011; Grant and Dickens, 2011; Hill et al., 2005; Lu et al., 2007; Siju et al., 2010; Syed and Leal, 2007, 2009). Responses of these neurons have been attributed to the activation of at least three conserved ORs including OR2, OR8 and...
The modulation of insect behavior for the purpose of controlling the spread of infectious diseases has been the task of a few insect repellents for which the mechanistic modes of action on odorant receptors (ORs) are unclear. Here, we study the effects of the repellents DEET and IR3535, and a novel OR coreceptor (Orco) agonist on odorant-evoked currents in Xenopus oocytes expressing two subtypes of Aedes aegypti ORs (AaORs). We show that DEET and IR3535 behave as insurmountable antagonists of ORs and that modulation of OR activity is not restricted to antagonism and agonism, but also includes synergism. This knowledge of the molecular mechanisms underlying OR blockade, activation and hyperactivation will be fundamental to the development of novel strategies for the control of mosquito behavior.
OR10 (Lu et al., 2007; Bohbot and Dickens, 2009; Wang et al., 2010; Bohbot and Dickens, 2010; Pelletier et al., 2010; Hughes et al., 2010; Bohbot et al., 2010). Due to the narrow tuning properties of these receptors, these behaviorally salient odorants are assumed to bind to one or multiple orthosteric sites. Until recently, orthosteric sites on the odorant-binding subunit or on Orco were unknown (Elmore et al., 2003; Neuhaus et al., 2005; Nichols et al., 2011). Now, a synthetic Orco agonist called VUA1 (hereafter called OrcoRAM1 for Orco Receptor Activator Molecule 1) has provided additional insight as to its capability for ion transport that is mediated by an allosteric site (Jones et al., 2011). However, it should be noted that it is unknown whether each subunit of the OR complex form functionally independent ion channel pore(s) or that multiple subunits are required to form a single channel.

In Aedes aegypti, the octenol receptor is enantioselective toward the \((R)-(\text{S})\)-enantiomer (hereafter referred to octenol) (Fig. 1) over the \((S)-(\text{R})\)-enantiomer of 1-octen-3-ol (Bohbot and Dickens, 2009) and largely accounts for the specificity of the physiological response observed in vivo (Grant and Dickens, 2011). In Culex pipiens quinquefasciatus, CqOR10-CqOrco discriminates between 3-methylindole (also known as skatole, Fig. 1) and indole (Hughes et al., 2010). When expressed in Xenopus oocytes, AaOR8-AaOrco and CqOR10-CqOrco exhibit sensitivity to these compounds in the high nanomolar range, which is matched by pheromone receptors of other insects in the same expression system (Nakagawa et al., 2005; Wang et al., 2010; Wanner et al., 2010, 2007), thus supporting the idea that octenol and skatole interact with orthosteric sites located on the AaOR8 and CqOR10 subunits, respectively.

The operative mechanism of insect repellent-OR interactions is unclear as these compounds exhibit a wide range of non-selective, selective, agonistic and antagonistic effects (Bohbot and Dickens, 2010; Bohbot et al., 2011; Ditzen et al., 2008; Jones et al., 2011; Liu et al., 2010; Xia et al., 2008). In a previous study, we used AaOR2-AaOrco and AaOR8-AaOrco to parse out which of the two receptor components might be targeted by insect repellents (Bohbot and Dickens, 2010). Selective effects, such as the one displayed by DEET (Fig. 1) and 2-undecanone, suggested that the sensing component of the OR complex was targeted; the non-selective effects of IR3535 (Fig. 1) and picaridin were ORx independent and therefore assumed to be mediated by Orco (Bohbot and Dickens, 2010). In support of this hypothesis, DEET was recently shown to interact directly with the Drosophila ligand-binding subunit OR59B in vivo (Pellegrino et al., 2011).

We have previously reported the agonist and antagonist properties of multiple insect repellents (Bohbot and Dickens, 2010; Fig. 1. Structure of the receptor ligands. Structures of odorants, insect repellents, OrcoRAM1 and OrcoRAM2 used in experiments. OrcoRAM1 has the nitrogen atom on the pyridine ring in the meta position whereas the OrcoRAM2 nitrogen of the pyridine ring is in the para position.)
Bobbot et al., 2011). In our current study, we provide a more detailed pharmacological analysis of DEET and IR3535 on octenol (AaOR8-AaOrco) and skatole (AaOR10-AaOrco) receptors, expressed in Xenopus oocytes. Knowledge of the molecular mechanisms underlying the divergent modes of OR blockade and potentiation will be fundamental to the development of novel strategies for the control of mosquito behavior based on modulation of the olfactory input.

2. Materials and methods

2.1. Expression of ORs in Xenopus laevis oocytes and two-microelectrode voltage-clamp electrophysiological recordings

The protocols used in this study have been described elsewhere (Bobbot and Dickens, 2009). Briefly, AaOR10, AaOrco and AaOR8 cRNAs were injected into Ringer's mMESSAGE mMACHINE SP6 kit (Ambion) and linearized psiP64DV expression vectors as template (Dr. L.J. Zwiebel, Vanderbilt University). Mature oocytes (stage V–VI) were treated for 35 min at room temperature with 2 mg/ml collagenase (SIGMA, C6895) in washing buffer (96 mM NaCl, 2 mM KCl, 5 mM MgCl2 and 5 mM HEPES [pH 7.6]). Following microinjection with 27.6 ng AaOr coRNAs, oocytes were incubated in washing buffer supplemented with 5% diazolase horse serum, 50 mg/ml tetracycline, 100 mg/ml streptomycin and 550 mg/ml sodium pyruvate for four to five days. Whole-cell currents were recorded from injected oocytes using the two microelectrode voltage-clamp technique (Nakagawa et al., 2005; Sumikawa et al., 1981). A holding potential of ~80 mV was maintained by an OC-725C oocyte clamp (Warner Instruments) during recording sessions. Oocytes were exposed to odorants alone or to odorant/drug combinations in 1% DMSO for 8 s. Current was allowed to return to baseline between drug administrations. Data acquisition and analysis were carried out with Digidata 1440A and pCLAMP10 software (Axon Instruments). Dose response data were analyzed using GraphPad Prism 5.

2.2. Drug application

The data illustrated in Fig. 2 were obtained using the following protocol: the oocytes were continuously perfused with Ringer’s solution and exposed to 8 s long administrations of odorant alone or a combination of odorant and insect repellent prepared in Ringer’s solution supplemented with 1% DMSO. For the equilibration experiment (Fig. 3), the oocytes were first perfused in Ringer’s solution in the absence of insect repellent and exposed to an 8 sec stimulation of cognate odorant at 10^-7 M. Two minutes following this stimulation, the perfusion solution was again perfused to Ringer’s solution supplemented with 10^-2 M insect repellent. The ensuing inhibition was then challenged with 8 s administration of a mixture of odorants and 10^-2 M DEET or IR3535. Finally, the oocytes were perfused with Ringer’s solution for 2 min and stimulated with an 8 s burst of 10^-3 M odorant. In all other experiments, oocytes were continuously perfused with insect repellent-free Ringer’s solution. Odorant and insect repellents (alone or in combination) were delivered as 8 s administrations.

2.3. Chemicals

Skatole (99%) was obtained from Alfa Aesar, Ward Hill, MA, USA. (R)-(−)-1-octen-3-ol [99.6% (R) form] was custom synthesized by Bedoukian Research, Inc. The repellents used in this study were DEET, N,N-diethyl-3-methylbenzamide [99.2%, Aldrich Chemical Co., Milwaukee, WI, USA] and IR3535 [3-(N-butyl-N-acetyl)-aminopropanionic acid ethyl ester (>95%, Merck, Rahway, NJ, USA)]. VAIA1 – OrcoRAM1 [N-(4-ethylphenyl)-2-(4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl) thio]acetamide] was provided by Dr. L.J. Zwiebel, Vanderbilt University and OrcoRAM2 [N-(4-ethylphenyl)-2-(4-ethyl-5-(4-pyrididy)-4H-1,2,4-triazol-3-yl)thio]acetamide] (CAS no. 525582-84-7) was obtained from Innovapharm Ltd., Kiev, Ukraine.

3. Results

3.1. DEET and IR3535 are antagonists of the skatole response

By definition, receptor antagonists have no efficacy of their own while blocking agonist responses due to their affinity for orthosteric or allosteric sites on the receptor (Gaddum et al., 1955). In order to eliminate any contribution to the response elicited by partial agonists like DEET and IR3535 (Bobbot and Dickens, 2010), we decided to use two divergent AaORs for which DEET and IR3535 have no activating effects, while blocking odorant-evoked responses. The inhibition of AaOR8-AaOrco responses to octenol by DEET and IR3535 satisfies this requirement. However, DEET’s agonist effects on AaOR2-AaOrco precluded its use herein (Bobbot and Dickens, 2010; Bobbot et al., 2011). Therefore, AaOR2-AaOrco was substituted with AaOR10-AaOrco, which was not activated by either DEET or IR3535 (Supplemental Fig. 1) and exhibited remarkable sensitivity toward skatole (EC50 = 109 nM) (Fig. 2). Supplied with two odorant-specific Aedes receptor complexes and two true antagonists (i.e., without any intrinsic agonist activity), we proceeded to explore the mechanism of DEET and IR3535 antagonism in greater detail.

3.2. DEET and IR3535 are insurmountable antagonists

We established the concentration–response relationships of AaOR8-AaOrco and AaOR10-AaOrco for their natural ligands in the presence of increasing amounts of DEET and IR3535. DEET and IR3535 did not affect the maximal response (Fig. 2A) but caused a right shift of the octenol–response curve (Fig. 2B); indicative of surmountable antagonism (Gaddum et al., 1955). In contrast to AaOR8-AaOrco, DEET and IR3535 produced a progressive diminution of the maximal response of AaOR10-AaOrco (Fig. 2C) with a concomitant dextral displacement of the skatole response (Fig. 2D); consistent with insurmountable antagonism (Neubig et al., 2003).

Competitive antagonists occupy the agonist-binding site of a receptor (orthosteric site) thereby shifting the agonist concentration–response curve to the right without reducing agonist efficacy (Neubig et al., 2003). In the case of a competitive antagonist, a plot of this shift versus the concentration of antagonist will produce a straight line with a slope of 1.0 known as Schild regression (Arunlakshana and Schild, 1959). DEET and IR3535 did not equally displace octenol potency at all concentrations (Fig. 2B), and Schild plots for AaOR8-AaOrco yielded slopes less than 1.0 (Supplemental Fig. 2). These results challenged the notion that OR8 antagonism by DEET/IR3535 was surmountable. Receptor reserve may mask the effects of insurmountable antagonists (Neubig et al., 2003). In this situation, a full agonist yields maximal response without activating all the receptors present on the cell surface.

In order to ensure complete receptor occupancy (suppressing potential receptor reserve in our system), OR-expressing Xenopus oocytes were exposed to a continuous stream of 10^-2 M DEET or IR3535 solutions. The resulting receptor blockades were challenged with 8 s administrations of odorant ranging from 10^-7 to 10^-4 M (Fig. 3A). Control for receptor expression was tested with an initial administration of 10^-7 M octenol or skatole in Insect Repellent-free Ringer’s solution (Fig. 3A). The continuous presence of insect repellents in the perfusion solution yielded a reduction of the maximal responses (Fig. 3B) along with significant dextral displacement of the concentration–response curves (Fig. 3C), characteristic of insurmountable antagonism. The maximal response values for each concentration–response curve were statistically compared using a one-way ANOVA followed by Tukey’s post test (data not shown). Despite a trend toward reduction, the maximal response of AaOR8-AaOrco under 10^-3 M IR3535 treatment did not show a statistically significant difference with the control treatment (no insect repellent control at 10^-7 M skatole and 10^-4 M octenol). For all other treatments, the differences were highly significant. IR3535 also increased repolarization duration for both AaOR8-AaOrco and AaOR10-AaOrco.

Except for AaOR10-AaOrco versus DEET, these inhibitions were lifted by 10^-6 M agonists and higher concentrations (Fig. 3A and B). DEET was a more effective antagonist than IR3535. In particular, DEET completely blocked AaOR10-AaOrco at all tested agonist concentrations (Fig. 3A and B). We then asked whether this antagonism could be reversed by first washing the oocytes for 2 min with insect repellent-free Ringer’s solution and exposing the
receptors to $10^{-7}$ M octenol or skatole. Only octenol was able to evoke a small AaOR8-AaOrco current (10.5% of the initial stimulation, Fig. 3A) after being inhibited by IR3535. Beside this exception, OR8 and OR10 remained blocked.

3.3. Octenol potentiates AaOR8-AaOrco responses to OrcoRAM2

At a concentration of $2 \times 10^{-4}$ M, OrcoRAM2 evoked a weaker response (ca. 2.5 times less) than OrcoRAM1 from AaOrco alone.
Supplemental Fig. 3. We established OrcoRAM2 concentration–response curves for AaOrco alone and AaOR8-AaOrco (Fig. 4A). The highest maximum response was observed between OrcoRAM2 and AaOrco alone (Fig. 4A). A non-linear regression analysis of the AaOrco response yielded a steep slope coefficient close to eight (standard Hill slope factors typically range between 0.5 and 2).

AaOR8-AaOrco pre-exposed for 8 sec to 10^{-7} M octenol displayed a stronger activation pattern than naive (i.e., not exposed to octenol) receptor assemblies. Since octenol pre-exposure appeared to potentiate the efficacy of OrcoRAM2, we tested the combined effect of OrcoRAM2 and octenol on the AaOR8-AaOrco complex. A mixture of 10^{-7} M octenol and 2 \times 10^{-4} M OrcoRAM2 evoked

Fig. 3. DEET and IR3535 are insurmountable antagonists. (A) AaOR8-AaOrco and AaOR10-AaOrco responses to odorants under continuous perfusion with 10^{-2} M DEET or IR3535. Oocytes were perfused with insect repellent-free Ringer’s solution and stimulated with octenol or skatole prior to and following equilibration in Ringer’s supplemented with 10^{-2} M DEET or IR3535. (B) DEET and IR3535 shifted the odorant concentration–response curve of both receptors to the right (horizontal arrow) with a concomitant decrease in maximal response (vertical arrow). (C) EC_{50} ranking profile of AaOR10-AaOrco and AaOR10-AaOrco under continuous inhibition by DEET or IR3535 and exposed to increasing concentrations of odorants. Higher odorant concentrations significantly affected OR sensitivity (one-way ANOVA followed by Tukey’s post test; P < 0.001). EC_{50} (AaOR10-AaOrco) could not be determined due to the complete OR blockade by DEET. Results are shown as means ± SE, n = 5–6.
a greater response than the sum of the individual responses produced by octenol and OrcoRAM2 indicating that OrcoRAM2 and octenol act synergistically (Fig. 4B).

3.4. DEET and IR3535 do not inhibit Orco activation

In the following experiment, we used OrcoRAM2, the para-substituted pyridine analogue of OrcoRAM1 (commercially discontinued at time of this study), as an Orco agonist (Fig. 1) to further investigate the mode of action of DEET and IR3535. The selective and non-selective inhibition patterns of OR2, OR10 and OR8 by insect repellents seen here and elsewhere (Bohbot and Dickens, 2010; Bohbot et al., 2011) suggested that these inhibitions were either mediated via the ligand-binding subunit or via Orco. We used $10^{-2}$ M DEET and IR3535 to challenge AaOrco’s activation by $2 \times 10^{-4}$ M OrcoRAM2. Neither DEET or IR3535 diminished the efficacy of OrcoRAM2 (Fig. 5).

4. Discussion

As in Culex, AaOR10-AaOrco is highly sensitive to skatole, an analogue of indole. The advantage of using AaOR10 over AaOR2 was...
that neither insect repellent, DEET or IR3535, activated AaOR10. This allowed us to focus strictly on the antagonistic effects of the repellents on two receptor complexes for which ligands of ecological significance are known and likely bind to an orthosteric site (Fig. 6A.1). The discovery that Drosophila OR59B (Pellegrino et al., 2011) and AaOR10-AaOrco are targeted by insect repellents provides additional evidence that these compounds modulate OR sensitivity.

While we recognize that ligands may induce tertiary changes upon binding, our data suggest that ORs spontaneously adopt multiple conformational states in a dynamic equilibrium (Monod et al., 1965). In this model, the open and closed states exhibit various binding sites characterized by specific ligand affinities. Upon binding, a ligand shifts the equilibrium by favoring a particular state. In this study, the odorant alone (i.e. octenol or skatole), OrcoRAM2 alone or a mixture of both compounds activated the OR complex (Fig. 6A.1). OrcoRAM2 was also a strong agonist of Orco when this subunit was expressed singularly without the ligand binding subunit (Fig. 6B). Compared to the “induced-fit model” (Changeux, 2011), the “selection model” provides a mechanistic explanation for the low level of spontaneous OR activity observed in the absence of odorants (Sargsyan et al., 2011; Sato et al., 2008; Wicher et al., 2008).

We initially showed that DEET and IR3535 displayed OR-dependent surmountable (dextral shift of the concentration—response curve only) and insurmountable antagonism (concomitant dextral shift of the concentration—response curve and partial

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**Fig. 5.** DEET and IR3535 do not inhibit OrcoRAM2-activated AaOrco. (A) Response traces of AaOrco to $2 \times 10^{-4}$ M OrcoRAM2 alone and in combination with $10^{-3}$ M DEET or IR3535. (B) Normalized responses of AaOrco in response to $2 \times 10^{-4}$ M OrcoRAM2 alone, and to a mixture of $2 \times 10^{-4}$ M OrcoRAM2 and $10^{-3}$ M DEET or IR3535. All treatments yielded statistically equivalent responses ($P < 0.05$, ANOVA test with Tukey posttest). Results are shown as means ± SE, n = 5–6.
depression of the maximal response). However, the antagonist equilibration experiment supported the insurmountable nature of DEET and IR3535 inhibition. Insurmountable antagonism can be explained by two distinct mechanisms: orthosteric and allosteric interactions. In the “kinetic” interpretation, DEET and IR3535 act as competitive antagonists, i.e., they form long-lasting complexes with the orthosteric site of the receptor but have no intrinsic activity on their own (Fig. 6A.2). This is consistent with the equilibration experiments where receptor blockade may be explained by tight or covalent chemical bonds between insect repellents and the receptors. This phenomenon was pronounced with DEET, resulting in an irreversible blockade of AaOR10-AaOrco. IR3535 showed a similar yet less pronounced (reversible blockade) inhibition as it increased current duration following odorant stimulation. Alternatively, DEET and IR3535 may interact with one or more allosteric sites. In this model, antagonist binding stabilizes the closed state conformation of the receptor, which has little or no affinity for the odorant or is prevented from being activated (Fig. 6A.3). Based on the remarkable sensitivity and specificity of OR8 and OR10 for their cognate ligands, and due to the heteromeric nature of ORs, our present findings are more compatible with a model in which DEET and IR3535 interact with an allosteric site on the receptor complex (Fig. 6A.3).

DEET and IR3535 did not block OrcoRAM2-mediated responses of AaOrco alone suggesting that the recognition site for the repellent is not located on Orco (i.e., DEET and IR3535 have a recognition site on the sensing subunit) (Bohbot and Dickens, 2010; Pellegrino et al., 2011), or that Orco has no affinity for the insect repellents when bound to OrcoRAM2. These results suggest that allosteric sites for DEET and IR3535 may emerge as a result of heteromeric complex formation (Fig. 6A.3).

The Hill slope factor of AaOrco in response to OrcoRAM2 was significantly greater than 1.0 indicating that Orco may form homomeric complexes possessing more than one binding site for this ligand (Christopoulos and Kenakin, 2002) (Fig. 6B). Homomeric formation of the Drosophila Orco has been reported in heterologous
expression systems (Benton et al., 2006; Neuhaus et al., 2005). OrcoRAM2 was significantly more potent on AaOrco alone (Fig. 6B) than on the AaOR8-AaOrco complex (Fig. 6A.1) suggesting that the latter has little affinity toward OrcoRAM2 alone. OrcoRAM2’s potency had also been shown to correlate with receptor heteromerization (Jones et al., 2011). This is an important aspect for the development of future insect repellents, as allosteric modulation of ORs comprises both potency and efficacy elements. In this regard, the enhanced response of AaOR8-AaOrco evoked by a combination of octenol and OrcoRAM2 suggested synergistic activity (Fig. 6A.1). A possible mechanism accounting for this phenomenon is that odorant-binding may select the open state conformation which concomitantly exhibits high affinity for OrcoRAM2. Odorant/allosteric agonist mixtures may therefore be exploited for the development of future insect repellent formulations aimed at confusing insect behaviors by hyperactivating ORs.

5. Conclusions

The study of OR modulation by insect repellents is in its early days. Several molecular modes of action for DEET have been proposed (Davis and Sokolove, 1976; Dogan et al., 1999; Syed and Leal, 2008; Xia et al., 2008; Ditzen et al., 2008; Bobbot and Dickens, 2010). Pellegrino et al. (2011) have recently substantiated the view that insect repellents exert a dual role on ORs: activation and inhibition (Bobbot and Dickens, 2010; Bobbot et al., 2011). In this study, we identify the skatole receptor as being targeted by DEET and IR3535, expanding the portfolio of insect ORs modulated by insect repellents. We show that DEET and IR3535 behave as insurmountable antagonists of the heteromeric receptor complex. While the nature of DEET and IR3535 antagonism is better understood, the operative mechanism for their mode of action remains unknown. We have shown that ORs, odorant ligands and drugs interact in complex ways leading to multiple effects on receptor activation. It will be crucial to demonstrate whether the synergistic effects observed in this study are translated at higher levels of biological organization. Furthermore, our study demonstrates that OR modulation is both a function of subunit composition (homomeric versus heteromeric) and ligand environment (odorants, insect repellents and OrcoRMs).

Acknowledgments

The authors are grateful to Drs. Jason Pitts (Vanderbilt University), Thomas Heinbockel and Ze-Jun Wang (Howard University) for their critical reading of the manuscript. We also thank Dr. Laurence Zwiebel for providing the AaOR10 for their critical reading of the manuscript. We also thank Drs. Laurence Zwiebel for providing the AaOR10 and Thomas Heinbockel and Ze-Jun Wang (Howard University) for their critical reading of the manuscript. We also thank Drs. Laurence Zwiebel for providing the AaOR10 and Thomas Heinbockel and Ze-Jun Wang (Howard University) for their critical reading of the manuscript. We also thank Drs. Laurence Zwiebel for providing the AaOR10 and Thomas Heinbockel and Ze-Jun Wang (Howard University) for their critical reading of the manuscript.


