Evaluation of Novel Insecticides for Control of Dengue Vector *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT Insecticides are one of the major tools for controlling vector populations and for reducing the transmission of human pathogens. However, there are few new insecticides being developed and marketed for vector control. Herein, we report on the toxicity of six novel insecticides to both adult and larval *Aedes aegypti* (L). and the toxicity of three novel insect growth regulators (IGRs) to larvae. Four insecticides were highly or moderately toxic to larvae with LC_{50} values of 16 (chlorfenapyr), 70 (hydramethylnon), 79 (indoxacarb), and 84 ng/ml (imidacloprid). Diafenthiuron and chlorfenapyr were moderately toxic to adult mosquitoes with LC_{50} values of 13 and 92 ng/cm^2, respectively. Imidacloprid was strongly synergized by piperonyl butoxide (PBO) in *Ae. aegypti* adults, suggesting that neonicotinoids are intrinsically very toxic to adult mosquitoes (in the absence of detoxification). The effect of PBO on the toxicity in adults and larvae was considerably different, both in terms of the insecticides that were synergized (or antagonized for chlorfenapyr versus adults) and in terms of the degree of synergism. This result implies that the cytochrome P450s involved in metabolism of these insecticides are different between adults and larvae. Pyriproxyfen was confirmed as a potent IGR (EC_{50} of 0.0017 ng/ml) for mosquitoes, although tebufenozide lacked activity. The potential for use of these materials in mosquito control is discussed.

KEY WORDS cytochrome P450 monooxygenases, mosquito control, new insecticides, dengue, *Aedes aegypti*

MOSQUITOES ARE THE VECTORS of important human pathogens, including the etiologic agents of malaria, dengue, filariasis, yellow fever, and encephalitis. With the introduction of synthetic organic insecticides for vector control in the 1940s and 1950s, many areas of the world saw a large decrease in the prevalence of these diseases. However, the incidence of many vector-borne pathogens is increasing. Malaria infections are increasing, with 300–500 million clinical cases annually (Martinez 1998) and ≈2.5 million annual deaths (Butler 1997). Dengue viruses cause more human mortality and morbidity than any other arthropod-transmitted viral illness and rank second only to malaria among mosquito-transmitted infections. Currently, 2.5 billion people are at risk and ≈500,000 cases occur annually (Gubler 2002). One of the key factors leading to the rise in morbidity and mortality from vector-borne infections is resistance of mosquito vectors to insecticides.

Vector control efforts have been successfully used for decades to combat human disease. The use of insecticides to kill vectors often has been the only feasible method of disease control. Control programs dependent primarily on insecticides have had notable successes. For example, wide-scale house spraying of DDT in the 1950s and 1960s dramatically reduced malaria prevalence in Asia (Phillips 1983), and the Onchocerciasis Control Program's aerial spraying of temephos in riverine black fly breeding sites in West Africa nearly eliminated river blindness from certain regions during the 1970s and 1980s (Curtis 1989). In 1947, the Pan American Health Organization, armed with DDT, initiated a campaign in the Western Hemisphere to eradicate *Ae. aegypti.* By 1972, *Ae. aegypti* had been eradicated from 73% of the land area and 19 countries (Schliesman and Calheiros 1974, Gubler 1989). Eventually, DDT resistance was recognized as a serious problem (Brown and Pal 1971), and the campaign ended in 1972 before eradication goals were achieved.

Despite the unquestionable utility of insecticides in reducing the transmission of human pathogens, there are few new insecticides being developed and commercialized for vector control. This is because the high cost of insecticide discovery, the recent downsizing that the agrochemical industry has undergone, and the "low profitability" of the vector control market (i.e., the countries most in need of new insecticides for vector control have very limited resources to purchase insecticides). As a consequence of these factors, coupled with the evolution of resistance in vector species such as *Ae. aegypti* (Ponlawat et al. 2005), few insecticides remain available for vector control (Gratz and Jany 1994, Brogdon and McAllister 1998), and very

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few new insecticides are being offered as replacements.

Worldwide, mosquito control relies primarily on pyrethroids (e.g., permethrin, resmethrin, and phenothrin), organophosphates (e.g., temephos and chlorpyrifos), carbamates (e.g., propoxur and carbosulfan), insect growth regulators (IGRs, primarily methoprene), and biologicals (*Bacillus thuringiensis israelensis* and *Bacillus sphaericus*). However, loss of efficacy (because of resistance) and/or cost for some of these materials makes them unsuitable for mosquito control, especially in areas of the world that are subjected to the greatest human health threats from mosquitoes. Pyrethroid-treated bed nets can be an effective means for controlling malaria vectors and preventing disease (Curtis et al. 2003), but over-reliance on a single class of insecticides for bed nets is likely to lead to the evolution of resistance. Thus, as was recently pointed out (Zaim and Guillet 2002), there is an urgent need for alternative insecticides to control the vectors of human diseases.

Several new and promising insecticides have been developed for use against agricultural pests over the past several years: chlorfenapyr (Lovell et al. 1990), diafenthiuron (marketed in 1990; Tomlin 2003), hydramethylnon (marketed in 1980; Tomlin 2003), imidacloprid (Elbert et al. 1990), indoxacarb (marketed in 2000; Tomlin 2003), spinosad (marketed in 1997; Tomlin 2003), pyriproxyfen (registered in 1989; Tomlin 2003), and tebufenozide (Heller et al. 1992). These insecticides have a variety of mechanisms of action. Chlorfenapyr is a protoxin requiring activation, via cytochrome P450 monooxygenases, to exert its toxic effects, via uncoupling of oxidative phosphorylation (Black et al. 1994). Diafenthiuron has been implicated as an IGR that inhibits chitin synthesis (Hajjar and Casida 1978). Hydramethylnon is relatively slow acting and kills insects by inhibition of mitochondrial electron transport (Hollingshaus et al. 1984, Hollingshaus 1987). Imidacloprid is a promising new insecticide that interacts with the nicotinic acetylcholine receptor (Elbert et al. 1990, Bai et al. 1991). Indoxacarb (DPX-MP062) is a recently introduced oxadiazine insecticide with activity against a wide range of pests (Harder et al. 1996), including house flies (Sugiyama et al. 2001). In insects, indoxacarb seems to be decarbomethoxylated to DCJW by an esterase/amidase (Wing et al. 1998). Several studies have demonstrated that DCJW is effective at blocking sodium channels (Wing et al. 1998, 2000; Lapied et al. 2001, Tsurubuchi et al. 2001, Tsurubuchi and Kono 2003, Zhao et al. 2003), and it is more effective than indoxacarb at this target site (Wing et al. 2000, Tsurubuchi et al. 2001, Narahashi 2002, Tsurubuchi and Kono 2003). Spinosad acts primarily at the nicotinic acetylcholine receptor with a secondary site of attack possibly being GABA receptors (Salgado 1997). Pyriproxyfen and tebufenozide are IGRs that act as juvenile hormone or ecdysone agonists, respectively (Dhadialla et al. 1998).

Although the efficacy of a very limited number of these compounds has been reported against mosquitoes (references in *Results* and *Discussion*), many have not. In addition, reports on the insecticidal activity of some of these materials focused only on one life stage and in some cases without comparison to materials with known efficacy. Herein, we report on the toxicity of six insecticides (relative to the highly potent insecticide permethrin) to adult and larval *Ae. aegypti.* We chose *Ae. aegypti* because it is an important vector of human pathogens and because information from this species should be applicable to other mosquito species. Given that P450 monooxygenases are important in the metabolism of numerous insecticides (Hodgson 1985), we conducted assays in the presence and absence of the P450 inhibitor piperonyl butoxide (PBO). We also investigated the toxicity of three novel IGRs (pyriproxyfen, diafenthiuron, and tebufenozide) relative to a standard IGR (methoprene) currently used for mosquito control. The potential for these materials in mosquito control and as new tools for reducing the incidence of dengue infections is discussed.

Materials and Methods

Ae. aegypti. The ROCK (insecticide-susceptible) strain of *Ae. aegypti* was obtained from the University of Massachusetts and was used for all experiments. Eggs were hatched by placing a square of paper with eggs in a flask filled with 750 ml of distilled water and held under vacuum for 45 min. The vacuum was then released, and 38 mg of larval diet (3.6 g of brewer's yeast [MP Biomedicals, Irvine, CA] and 3.3 g of lactalbumin [St. Louis, MO]) were added to the water. The hatched larvae were held overnight in the flask, and then 200 larvae were transferred to a 3.9-liter plastic tray (27 by 21 by 7 cm) with a ventilated lid containing ≈2 liter of distilled water. Larval diet was added to each tray according to the following regime: day 1, 75 mg; day 3, 38 mg; day 4, 75 mg; day 5, 113 mg; and day 6, 150 mg. Mosquitoes were reared in an environmental chamber set with a temperature profile representing a simulated summer day regime (ranging from 22 to 30°C) and 80% RH. Incandescent lighting was set to a crepuscular profile with a photoperiod of 14:10 (L:D) h, including 2 h of simulated dawn and 2 h of simulated dusk. Adults were held in a 60 by 60 by 60-cm screened cage and provided 10% sucrose ad libitum. Chicken blood (with 1% heparin) wrapped in pig intestine and warmed to 37°C was provided to adults twice a week. Eggs were collected on paper towels (Vasco brands, Elmira, NY) lining the rim of water containers. The papers with eggs were air dried at 27°C and 80% humidity for 24 h and stored in containers with 100% humidity for 3–30 d. Eggs were hatched under vacuum and larvae were reared in containers as described above.

Chemicals. Chlorfenapyr (99.7%) and hydramethylnon (99.1%) were obtained from American Cyanamid (Princeton, NJ), diafenthiuron (99%) was from Novartis (Greensboro, NC), imidacloprid (97.4%) was from Miles Inc. (Kansas city, MO, 97.4%), indoxacarb (DPX MP062) was from DuPont (Wilmington, DE), permethrin (94.7%, 40:60 *cis:trans*) was from ICI

Table 1. Toxicity of seven insecticides to fourth instars of *Ae. aegypti* after 72-h exposure

Insecticide	Insecticide			Insecticide + PBO			
	LC_{50} (95% CI)[a]	Slope (SE)	*n*	LC_{50} (95% CI)[a]	Slope (SE)	*n*	SR[b]
Chlorfenapyr	16 (12–20)	2.5 (0.5)	1400	17 (12–25)	1.8 (0.3)	1740	0.9
Diafenthiuron	120 (110–130)	3.1 (0.3)	720	33 (21–53)	2.5 (0.7)	600	3.6*
Imidacloprid	84 (49–140)	1.7 (0.4)	1270	11 (8–14)	1.5 (0.2)	1010	7.6*
Indoxacarb	79 (62–100)	3.2 (0.6)	950	10 (6–13)	0.8 (0.2)	300	7.9*
Permethrin	1.6 (1.5–1.8)	3.0 (0.3)	820	0.14 (0.10–0.18)	1.6 (0.3)	700	11*
Hydramethylnon	70 (49–99)	3.4 (1.0)	1100	35 (24–50)	1.6 (0.3)	490	2.3
Spinosad	160 (120–200)	2.2 (0.3)	960	110 (97–150)	1.6 (0.2)	550	1.4

[a] LC_{50} in units of nanograms per milliliter.
[b] Synergism ratio = LC_{50} of insecticide/LC_{50} of insecticide + PBO.
* Significantly different from 1.0 ($P \leq 0.05$).

Americas Inc. (Richmond, CA), PBO (90%) was from Aldrich (Milwaukee, WI), pyriproxyfen (99.5%) and methoprene (98%) were from Chem Services (West Chester, PA), spinosad (88%) was from Dow AgroSciences (Indianapolis, IN), and tebufenozide (99.7%) was from Rohm & Haas (Philadelphia, PA).

Larval Bioassays. Twenty early fourth instars of uniform size were placed in 140-ml waxed paper cups (Horwitz, Horseheads, NY) containing 99 ml of distilled water and 1 ml of insecticide (in acetone) solution (or 1 ml of acetone for controls). To evaluate the role of P450 monooxygenases in the activation or detoxification of the insecticides, bioassays were run as described above except that 1 ml of PBO solution (0.3 mg/ml) also was added. Preliminary experiments indicated this was the maximum sublethal concentration of PBO. Larvae were considered dead if they were unresponsive to touching with a probe or if they could not reach the surface of the water. Because of the slow-acting nature of some of these insecticides, mortality was determined after 72 h of exposure. Mortality did not change after this time for the insecticides listed in Table 1 (except diafenthiuron; see *Results* and *Discussion*).

IGR Bioassays. IGR bioassays were set up as described above for the larval bioassays, except that the cups were covered with fabric (to contain emerged adults) and a perforated plastic cap (to minimize evaporation). Emergence was determined 10 d after insecticide exposure as complete emergence had occurred in all controls at this time. PBO was not included in the IGR assays because it prevents adult emergence at the concentrations used.

Adult Bioassays. Adult mosquito bioassays were conducted in glass jars (230 ml, internal surface area of 180 cm^2) treated with 1 ml of insecticide solution (or 1 ml of acetone for the controls), which was evenly coated on the inner walls. To evaluate the role of monooxygenases in the activation or detoxification of the insecticides, bioassays were run as described above except that 1 ml of PBO solution (0.3 mg/ml) also was added. Acetone was allowed to evaporate for 30 min and then 10 adult females (2 to 3 d old) per glass jar were placed inside each jar, and the opening was covered with fabric. Adults in the glass jars had access to 10% sucrose solution from dental wicks. Adults were considered dead if they were ataxic. Because of the slow acting nature of some of the insecticides tested, mortality was determined after 48 h of exposure.

Analysis. At least five concentrations were used for each bioassay. Every bioassay was held at 25°C (≈60% RH) and was replicated a minimum of five times. Bioassay data were pooled and analyzed by standard probit analysis (Finney 1971), as adapted to personal computer use (Raymond 1985) using Abbott's (Abbott 1925) correction for control mortality. LC_{50} values were judged as significantly different ($P \leq 0.05$) if the confidence intervals did not overlap.

Results and Discussion

Toxicities of permethrin and six novel insecticides to fourth instars of *Ae. aegypti* are shown in Table 1. Permethrin was the most toxic larvicide (LC_{50} = 1.6 ng/ml), followed by chlorfenapyr (16 ng/ml), and three moderately toxic (70–84 ng/ml) compounds (hydramethylnon, indoxacarb, and imidacloprid). Diafenthiuron and spinosad were the least toxic (LC_{50} values >100 ng/ml). Our results with imidacloprid agree with a previous study on *Culex quinquefasciatus* Say (Liu et al. 2004), but they disagree with a study on *Ae. aegypti* and *Ae. taeniorhynchus* that found imidacloprid to be highly potent (Song et al. 1997). Our results with spinosad agree with a report on *Cx. quinquefasciatus* (Liu et al. 2004), but disagree with a recent report (Bond et al. 2004) that found spinosad to be highly toxic to *Aedes* and *Anopheles* larvae. This difference may be because of the fact that these authors (Bond et al. 2004) used formulated spinosad (Tracer) rather than technical grade material. Our results indicate chlorfenapyr, hydramethylnon, indoxacarb, and imidacloprid warrant further field evaluations as larvicides because they are similar in toxicity to current mosquito control standards: organophosphate and carbamate insecticides (Priester and Georghiou 1980).

In the presence of the cytochrome P450 inhibitor PBO, permethrin was still the most toxic larvicide. Indoxacarb, imidacloprid, and chlorfenapyr were ≈100-fold less toxic than permethrin. Diafenthiuron and hydramethylnon were slightly toxic, whereas spinosad was least toxic. The largest synergism ratio was observed for permethrin (11-fold), followed by indoxacarb (7.9-fold), imidacloprid (7.6-fold), and

Table 2. Toxicity three IGR insecticides to fourth instars of *Ae. aegypti*

Insecticide	EC_{50} (95% CI)[a]	Slope (SE)	*n*
Methoprene	0.047 (0.022–0.110)	0.4 (0.1)	440
Diafenthiuron	0.14 (0.10–0.26)	1.4 (0.2)	340
Pyriproxyfen	0.0017 (0.0007–0.0041)	1.0 (0.3)	300
Tebufenozide	>10,000[b]		200

Toxicity was evaluated as percentage of adult emergence after 10 d.
[a] Effective concentration to cause 50% of treated larvae to fail to emerge as adults in units of nanograms per milliliter.
[b] Ten percent failed to emerge at a concentration of 10 μg/ml.

diafenthiuron (3.6-fold). The toxicity of chlorfenapyr was not significantly changed by PBO, which was surprising because this compound is thought to be bioactivated by P450s (Black et al. 1994).

The toxicities of three IGRs—diafenthiuron, pyriproxyfen, and tebufenozide—relative to methoprene are shown in Table 2. Pyriproxyfen was ≈28-fold more toxic than methoprene, suggesting this material holds promise for mosquito control, and this finding is consistent with previous studies (Schaefer et al. 1988, Kamimura and Arakawa 1991, Dell Chism and Apperson 2003, Satho et al. 2003). Diafenthiuron was threefold less toxic than methoprene. Tebufenozide was practically nontoxic, affecting only 10% of the larvae, even at a dose of 10 μg/ml. This is in agreement with 48-h larval bioassays that showed <50% kill at the highest concentration tested (2.5 mg/liter) for *Ae. aegypti* held at 20°C (Song et al. 1997). Overall, methoprene and pyriproxyfen are more toxic relative to the insecticides shown in Table 1. Thus, although IGRs are slower acting than other types of insecticides, they are remarkably potent.

The toxicity of seven insecticides to adult *Ae. aegypti* is shown in Table 3. Permethrin was highly toxic (LC_{50} = 0.29 ng/cm^2), diafenthiuron and chlorfenapyr were moderately toxic (LC_{50} = 13 and 92 ng/cm^2, respectively), and spinosad was slightly toxic (LC_{50} = 460 ng/cm^2), whereas hydramethylnon, indoxacarb, and imidacloprid had LC_{50} values of >6,300 ng/cm^2. Based on these results, it seems that diafenthiuron and chlorfenapyr hold promise for control of adult mosquitoes.

Diafenthiuron was 860-fold more toxic in the adult emergence assay, compared with the larval 72-h assay, which is consistent with an IGR (i.e., inhibition of chitin synthesis (Hajjar and Casida 1978) type of action. However, diafenthiuron was also toxic to larvae (72-h bioassay) and adults (48-h bioassay), suggesting diafenthiuron may have another mechanism of action (in addition to inhibition of chitin synthesis) responsible for the acute toxicity seen to larvae and adults.

In the presence of PBO, permethrin was still the most toxic insecticide to adults. However, diafenthiuron, imidacloprid, and to lesser extent spinosad were moderately toxic. Chlorfenapyr, indoxacarb, and hydramethylnon were practically nontoxic. PBO was strongly synergistic to imidacloprid and spinosad with a synergism ratio of >2000- and 63-fold, respectively. This suggests P450s greatly limit the toxicity of imidacloprid and spinosad in adult *Ae. aegypti* (although the role of PBO in facilitating penetration of these insecticides cannot be ruled out). Given the toxicity of imidacloprid + PBO, it would be useful to examine the toxicity of other neonicotinoids to see whether one could be found that was not so substantially detoxified by P450 monooxygenases. PBO also significantly synergized the toxicity of diafenthiuron and permethrin (Table 3). PBO results in antagonism of chlorfenapyr toxicity, consistent with the role of P450s in activation of this insecticide. The LC_{50} values of indoxacarb + PBO and hydramethylnon + PBO were >6,300 ng/cm^2, suggesting toxicity of these insecticides is not limited by P450-mediated detoxification.

The effect of PBO on the toxicity in adults and larvae was considerably different, both in terms of the insecticides that were synergized (or antagonized for chlorfenapyr versus adults) and in terms of the degree of synergism. This implies that the P450s involved in metabolism of these insecticides are different between adults and larvae.

In summary, our results indicate chlorfenapyr, hydramethylnon, indoxacarb, and imidacloprid warrant further field evaluations as larvicides, whereas diafenthiuron and chlorfenapyr hold promise for control of adult mosquitoes. Pyriproxyfen shows excellent activity as an IGR, and neonicotinoids should be further investigated for their potential to control adult mosquitoes. Given the urgent need for new and more effective approaches to dengue vector control, the

Table 3. Toxicity of seven insecticides to adult female *Ae. aegypti* 48 h after treatment

Insecticide	Insecticide			Insecticide + PBO			
	LC_{50} (95% CI)[a]	Slope (SE)	*n*	LC_{50} (95% CI)[a]	Slope (SE)	*n*	SR
Chlorfenapyr	92 (65–130)	1.4 (0.2)	330	>6,300[b]	1.8 (0.3)	1740	0.01*
Diafenthiuron	13 (10–18)	1.8 (0.4)	1060	3.7 (1.8–6.9)	0.6 (0.2)	440	3.5*
Imidacloprid	>6,300[c]			2.8 (1.9–4.2)	2.4 (0.6)	360	>2,000*
Indoxacarb	>6,300[c]			>6,300[c]			
Permethrin	0.29 (0.26–0.34)	2.7 (0.3)	540	0.11 (0.07–0.18)	1.6 (0.3)	730	2.6*
Hydramethylnon	>6,300[c]			>6,300[c]			
Spinosad	460 (410–610)	2.9 (0.2)	820	7.3 (4.5–9.7)	1.6 (0.3)	320	63*

[a] LC_{50} in units of nanograms per square centimeter.
[b] Zero percent mortality at 6.3 μg/cm^2.
[c] Less than 50% kill at 6.3 μg/cm^2.
*Significantly different from 1.0 ($P \leq 0.05$).

efficacy and feasibility of using these insecticides should be a high priority for the immediate future.

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