

# Evaluation of Indoxacarb, an Oxadiazine Insecticide for the Control of Pyrethroid-Resistant *Anopheles gambiae* (Diptera: Culicidae)

RAPHAEL N'GUESSAN,<sup>1,2,3</sup> VINCENT CORBEL,<sup>4</sup> JULIEN BONNET,<sup>4</sup> ALISON YATES,<sup>1</sup>  
ALEX ASIDI,<sup>1</sup> PELAGIE BOKO,<sup>2</sup> ABIBATOU ODJO,<sup>2</sup> MARTIN AKOGBÉTO,<sup>2</sup>  
AND MARK ROWLAND<sup>1</sup>

J. Med. Entomol. 44(2): 270–276 (2007)

**ABSTRACT** Owing to the spread of pyrethroid resistance in *Anopheles gambiae* s.s. (Diptera: Culicidae) and other vector mosquitoes, there is an urgent need to develop alternative insecticides to supplement the pyrethroids for malaria control. Indoxacarb is an oxadiazine insecticide initially commercialized by DuPont for control of agricultural pests. Performance against *An. gambiae* bearing *kdr* (pyrethroid and DDT resistance) or *Ace-1<sup>R</sup>* insensitive acetylcholinesterase (organophosphate and carbamate resistance) mechanisms was studied using larval and adult bioassays and a simulated experimental hut system (tunnel tests) that allows fuller expression of the behavioral responses to insecticide. Larval and adult bioassays (topical application and cone tests on treated netting) showed a standard probit dosage–mortality response and no evidence of cross-resistance to the *kdr* and *Ace-1<sup>R</sup>* resistance mechanisms. Toxic activity was slow compared with standard insecticides and additional mortality was observed. Indoxacarb induced no excitorepellency in adults. In tunnel tests, indoxacarb induced no inhibition of mosquito penetration or blood feeding through the holed netting, but it induced delayed mortality over 24–96 h. There was >90% mortality of the *kdr* strain on netting treated with the 500 mg/m<sup>2</sup> dosage, whereas permethrin at 500 mg/m<sup>2</sup> only killed 30% of this strain. A mixture of indoxacarb and pyrethroid showed neither synergism nor antagonism. The absence of cross-resistance to current insecticides indicates that indoxacarb has malaria vector control potential as larvicide or adulticide where mosquitoes are pyrethroid resistant.

**KEY WORDS** indoxacarb, mosquito, *Anopheles gambiae*, insecticide resistance

The scaling up of insecticide-treated nets (ITN) is a major element of international strategies to control malaria, particularly in sub-Saharan Africa (WHO 2002). Pyrethroids are the only class of insecticide currently recommended for use on ITNs (Zaim et al. 2000). In the last decade, pyrethroid resistance in anopheline mosquitoes has become widespread in western Africa, and pockets of resistance also have arisen in eastern, central, and southern Africa (Chandre et al. 1999, Hargreaves et al. 2000, Ranson et al. 2000, Etang et al. 2003). This development threatens to undermine current efforts to control malaria. In West Africa, alarm over the rapid spread of the *kdr* gene responsible for pyrethroid resistance in *Anopheles gambiae* s.s. (Diptera: Culicidae) (Martinez-Torres et al. 1998) was initially tempered by evidence from Ivory Coast that ITNs continue to reduce malaria transmission and morbidity in areas of *kdr* resistance (Henry et al. 1999, 2005).

However, recent trials of pyrethroids in experimental huts in southern Benin show that pyrethroid-treated nets and indoor residual spraying are largely ineffective against populations of *An. gambiae* carrying the *kdr* mechanism in that country (N'Guesan et al. 2007). In South Africa, the development of pyrethroid resistance in *An. funestus* Giles caused the failure of indoor residual house spraying (IRS) with deltamethrin (Hargreaves et al. 2000), and the outbreak was only brought under control after reversion to DDT spraying.

Organophosphate and carbamate insecticides are regarded as potential alternatives to pyrethroids (Najera and Zaim 2002) and have shown good efficacy as IRS treatments in some African situations (Najera et al. 1967; Fontaine et al. 1978), but not in others (Molineaux and Gramiccia 1980). Trials of ITNs treated with the organophosphate (OP) pirimiphos-methyl and the carbamate carbosulfan caused high mortality rates of pyrethroid-resistant *An. gambiae* in Ivory Coast (Kolaczinski et al. 2000). However, carbosulfan-treated nets may prove too hazardous for general use and select for insensitive acetylcholinesterase resistance in *An. gambiae* (Guillet et al. 2001, Corbel et al. 2003). Developing an alternative insecticide to

<sup>1</sup> London School of Hygiene and Tropical Medicine, Gates Malaria Partnership, London, WC1E 7HT, United Kingdom.

<sup>2</sup> Centre de Recherche Entomologique de Cotonou (CREC), Cotonou, Benin.

<sup>3</sup> Corresponding author, e-mail: raphael.n'guessan@lshtm.ac.uk.

<sup>4</sup> Institut de Recherche pour le Développement (IRD), Laboratoire de Lutte contre les Insectes Nuisibles (LIN), Montpellier, France.

which mosquitoes have no resistance has become a priority (Zaim and Guillet 2002). Over the last decade several new insecticides have been developed for use against agricultural pests, and some may have potential for malaria vector control. Indoxacarb is an oxadiazine insecticide produced by DuPont that shows low mammalian toxicity (Tomlin 2000) and contact and stomach activity against a wide range of insect pests, including Diptera and Lepidoptera (Wing et al. 1998, Sugiyama et al. 2001). Indoxacarb is activated by decarbomethoxylation to DCJW, which binds to sodium channels at a different site to pyrethroids and disrupts ion flow (Lapied et al. 2001).

To assess its potential for vector control, a series of phase I laboratory studies (WHO 2006) was undertaken against adults and instars of susceptible and insecticide-resistant strains of *An. gambiae*.

### Materials and Methods

**Mosquito Strains.** Four laboratory colonies were used: 1) *An. gambiae* Kisumu; a susceptible reference strain, originally from Kenya; 2) *An. gambiae* VKPER; pyrethroid resistant, fixed for the *kdr* gene, originally from the Kou Valley in Burkina Faso; 3) *An. gambiae* Yao; organophosphate and carbamate resistant, conferred by acetylcholinesterase site insensitivity (*Ace-I<sup>R</sup>*), originating from Yaokoffikro, Bouake, Côte d'Ivoire; and 4) *An. stephensi* Beech; a susceptible reference strain.

**Larval Bioassays.** Technical grade indoxacarb was provided by DuPont (Wilmington, NC). Insecticide solutions were prepared in ethanol, and tests were undertaken on late third and early fourth instars. Batches of 25 larvae were assayed in 99 ml of distilled water in plastic cups to which was added 1 ml of insecticide solution in ethanol. Four replicates per concentration over a seven to nine range of concentration conducted for each assay. Temperature was maintained at  $26 \pm 1^\circ\text{C}$ . Larval mortality was recorded after 24 h. Larvae were considered dead if they were unresponsive to touch or unable to reach the surface of the water. Data were analyzed using probit software (Raymond 1985).

**Topical Adult Applications.** Topical application allows estimation of intrinsic insecticide toxicity. A dilution series of indoxacarb in acetone was prepared, and tests were performed on batches of 50 unfed *An. gambiae* females, aged 2–5 d, per concentration. Mosquitoes were kept immobile over an ice block, and 0.1- $\mu\text{l}$  droplets of indoxacarb solution were applied to each thorax by using a glass micropipette; 0.1- $\mu\text{l}$  droplets of pure acetone served as controls. Females were supplied with honey solution and held for 24 h before scoring mortality.  $\text{LD}_{50}$  and  $\text{LD}_{95}$  values were estimated using probit analysis and expressed in nanograms per milligram of body weight. Indoxacarb was tested against pyrethroid-susceptible and -resistant strains.

**Cone Bioassay.** Formulated indoxacarb (15% SC) from DuPont was tested on polyester netting under WHO bioassay cones by using a range of concentra-

tions (50–1000  $\text{mg}/\text{m}^2$ ) and exposure times (3–24 min). Mortality was scored after 24, 48, and 72 h, and data were analyzed using logistic regression (STATA 6 software, Stata Corporation, College Station, TX) and probit analysis (Polo-PC, LeOra Software, Berkeley, CA).

**Irritability Test.** Two indoxacarb concentrations (100 and 500  $\text{mg}/\text{m}^2$ ) were tested for their irritant effect on Kisumu mosquitoes when applied to treated netting. Nonblood-fed *An. gambiae* females, aged 2–5 d, were introduced individually into plastic cones fixed to the netting, and after a settling period of 60 s, the time elapsing to the next take-off was recorded as the time to first take-off (Mouchet and Cavalié 1961). One hundred mosquitoes were tested individually on each concentration and control. Mosquitoes were grouped into geometric classes of time to first take off (0–1 s, >1–2 s, >2–4 s, >4–8 s, and >128–256 s). Probit analysis was used to calculate the time for 50% of mosquitoes to take off ( $\text{FT}_{50}$ ).

**Tunnel Tests.** The same range of indoxacarb dosages tested in cone bioassays were tested in tunnels against pyrethroid-resistant *An. gambiae* (VKPER strain); these tests were carried out in Benin. Permethrin dosages of a similar range were tested in tunnels against VKPER and Kisumu strains; these tests were carried out in France. The tunnel test is a laboratory system designed to allow expression of the behavioral interactions that occur between free-flying mosquitoes and ITN during experimental hut trials. Tunnel tests are carried out as a forerunner to hut trials, and they provide information on dosage-dependent repellency, blood-feeding inhibition, and mortality. The equipment consists of a square glass cylinder (25 cm in height, 25 cm in width, and 60 cm in length) divided into two chambers by means of a netting-covered frame that fits into a slot across the tunnel (WHO 2006). In one of the chambers, a guinea pig is housed unconstrained in an open-meshed cage and in the other chamber, 100 unfed female anopheline mosquitoes aged 2–5 d are released at dusk and left overnight. The netting is deliberately holed with nine 1-cm holes to give opportunity for mosquitoes to pass into the baited chamber. The next morning, the numbers of mosquitoes found live or dead, fed or unfed, in each chamber are scored. In our indoxacarb tests, live mosquitoes were removed from the chambers, given access to sugar solution, and monitored for delayed mortality up to 120 h. Data were analyzed using logistic regression (STATA 6 software).

**Synergy Tests.** Netting was treated with concentrations of indoxacarb or deltamethrin SC designed to give low mortality rates in 3-min cone bioassays and with a mixture of these to test for synergy or antagonism. Differences in observed mortality with the mixture and expected mortality from sequential application of the individual insecticides applied individually were examined using chi-square test.

Results

**Larval and Adult Topical Bioassays.** The summary results of probit analyses on the susceptible and resistant strains of *An. gambiae* in larval and adult topical tests are shown in Table 1. There were small and in some instances significant differences in mortality to indoxacarb at LC<sub>50</sub> level between pyrethroid- or OP-susceptible and -resistant strains (*kdr* and insensitive acetylcholinesterase *Ace-1<sup>R</sup>*), but the LC<sub>50</sub> ratios were always <2.

**Cone Test.** After 3-min exposure and scoring after 24 h, there was a positive dosage-mortality trend between 25 and 1,000 mg/m<sup>2</sup> (Fig. 1A). Significantly higher *An. gambiae* mortalities were observed 48 and 72 h postexposure at all dosages tested (*P* < 0.001), indicating delayed mortality with this insecticide. The dosages 250 mg/m<sup>2</sup> killed >80% but only 1,000 mg/m<sup>2</sup> killed 100%.

The mortality rates in the pyrethroid-resistant strain (VKPER) was not significantly different from those in the susceptible strain (Fig. 1B) at the dosages tested (*P* = 0.86), indicating an absence of cross-resistance of indoxacarb to *kdr*. The dosages 250 and 500 mg/m<sup>2</sup> killed >80% but only 1,000 mg/m<sup>2</sup> killed 100%.

Extending the exposure time from 3 min up to 24 min led to incremental increases in the proportion killed (Fig. 1C). The confidence intervals around the LD<sub>50</sub> estimates were smaller for the longer exposure periods (Fig. 1D).

**Irritability Test.** The time to first take-off as indicated by FT<sub>50</sub> did not differ between indoxacarb and control untreated netting (Table 2). The apparent 30% increase in irritability induced by the 500 mg/m<sup>2</sup> dosage was not significant. The majority did not take off during the observation period and the proportion not taking off did not differ between control and treated netting.

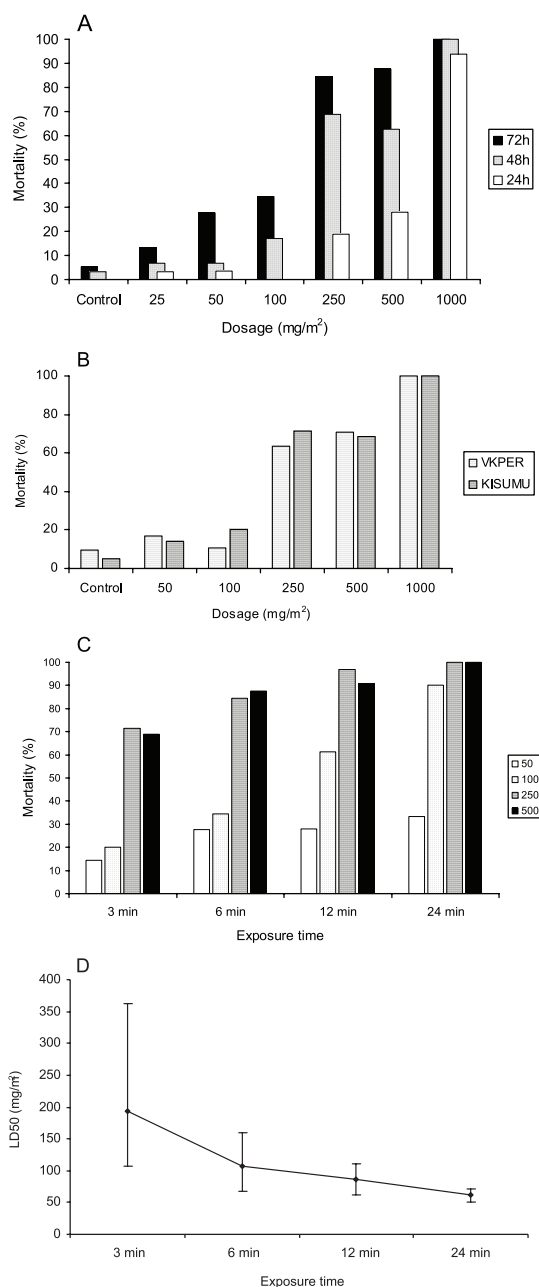
**Tunnel Tests.** Tunnel test results for indoxacarb and permethrin on netting is summarized in Fig. 2. The dosage-dependent mortality trend observed with indoxacarb in cone tests also was evident in tunnel tests. Mortality after 24-h holding was no different between control or any of the indoxacarb dosages, but between 24 and 96 h there was considerable treatment-induced mortality (Fig. 2A), e.g., >85% mortality at 96 h with netting treated with 500 mg/m<sup>2</sup> or higher concentrations. In the permethrin tests against VKPER, the mortality was never >30% even with the 500 mg/m<sup>2</sup> dosage (Fig. 2B). The mortality trend shown by permethrin against the pyrethroid-susceptible strain (Kisumu) was comparable with that shown by indoxacarb against VKPER.

In the indoxacarb tests, >80% of females penetrated the holed netting and 90% of these females went on to blood feed; there was no inhibition of penetration or blood feeding relative to the control (Fig. 2C). By contrast, in the permethrin tests against VKPER, there was a 10–20% insecticide-induced inhibition of penetration and up to 30% inhibition of blood feeding among the penetrating relative to the control (Fig. 2D). Many of those females that penetrated the in-

Table 1. Log dose-probit mortality data for indoxacarb larval assays and adult topical applications on *An. gambiae*

Strain	Larval bioassays				Topical application		
	Slope (SE)	LC <sub>50</sub> (95% CI) (mg/liter)	LC <sub>95</sub> (95% CI) (mg/liter)	RR <sub>50</sub>	LC <sub>50</sub> (ng/mg female)	LC <sub>95</sub> (ng/mg female)	RR <sub>50</sub>
Kisumu (S)	3.38 (0.07)	0.054 (0.044–0.066)	0.165 (0.114–0.241)				
VKPER ( <i>kdr</i> )	2.19 (0.15)	0.064 (0.057–0.072)	0.359 (0.289–0.474)	1.19 (0.88–1.62)	7.89 (6.68–9.30)	102.75 (73.84–156.11)	
Yao ( <i>Ace-1</i> )	2.53 (0.17)	0.103 (0.092–0.115)	0.459 (0.377–0.590)	1.91 (1.41–2.65)	13.84 (12.09–15.70)	86.39 (69.42–113.57)	1.75 (1.56–1.97)

RR<sub>50</sub> represents the resistance factor of *Kdr* or *Ace-1* strains relative to the Kisumu strain at LC<sub>50</sub> level.



**Fig. 1.** Efficacy of indoxacarb-treated netting across a range of dosages against *An. gambiae* in WHO cone assays. (A) Delayed mortality of the Kisumu strain after 3-min exposure to treated netting. (B) Mortality of Kisumu and VKPER strains 72 h after the initial 3-min exposure to treated netting. (C) Mortality of the susceptible Kisumu strain across a range of dosages and exposure times. (D) LD<sub>50</sub>s  $\pm$  CIs of the insecticide-susceptible Kisumu strain after exposure to treated netting for various exposure times.

doxcarb-treated netting and fed subsequently died (Fig. 2E), whereas fewer than 10% of VKPER females that penetrated the permethrin treated netting and

**Table 2.** Time to first take-off (FT<sub>50</sub>) of *An. gambiae* Kisumu exposed to indoxacarb treated netting in WHO irritability tests

	Dosage (mg/m <sup>2</sup> )	n	% not taking off (95% CI)	FT <sub>50</sub> (s) (95% CI) <sup>a</sup>	% induced irritancy at FT <sub>50</sub> level relative to control
Control	0	100	68 (59–77)	141 (55–1,978)	
Indoxacarb	100	100	56 (46–66)	134 (60–827)	4.5
Indoxacarb	500	100	50 (40–60)	98 (47–355)	30.1

<sup>a</sup> Time for 50% of the mosquitoes to take off as estimated using probit analysis.

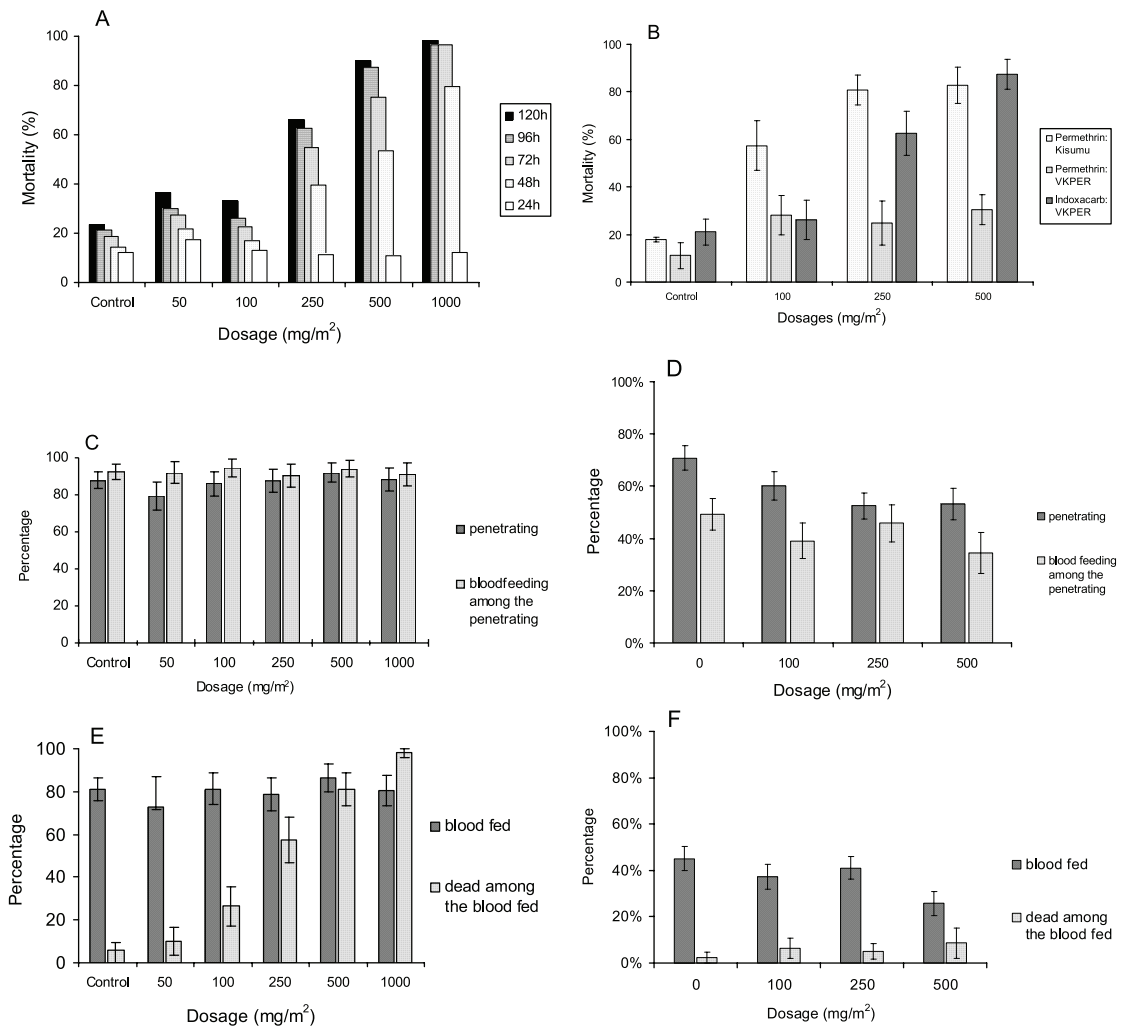
fed subsequently died (Fig. 2F). Penetration rates and blood-feeding rates were lower in the permethrin control than in the indoxacarb control; this finding was presumably due to differences in test conditions, permethrin being tested in Montpellier and indoxacarb in Benin.

**Synergy Tests.** The null hypothesis with the mixture was that the proportion surviving exposure to the first insecticide would be killed by the second insecticide at a rate indicated by the tests with the individual insecticide treatments. Mortality with the mixture did not differ from the null hypothesis; hence, the effect was additive rather than synergistic or antagonistic (Table 3).

## Discussion

A variety of larval and adult bioassay techniques for measuring intrinsic toxicity or natural behavioral responses to insecticide were consistent in showing no cross-resistance between indoxacarb and two types of mechanism that confer resistance to pyrethroids (*kdr*) or organophosphates and carbamates (insensitive acetylcholinesterase *Ace-1<sup>R</sup>*) in *An. gambiae*. As a larvicide, indoxacarb presumably acts through a combination of ingestion and contact action, as demonstrated previously in lepidopteran pests (Wing et al. 2000). In our studies indoxacarb showed larvicidal activity similar to some WHO-approved OP and pyrethroid larvicides used against susceptible mosquitoes (Corbel et al. 2004b), thereby warranting further evaluation in field situations where pyrethroid or OP resistance is common. As an adulticide on netting, indoxacarb required an application rate of  $\approx 250$ –500 mg/m<sup>2</sup> to induce high mortality. This dosage range is similar to that recommended for permethrin on nets. Although the indoxacarb-induced toxicity was delayed, the proportion of mosquitoes ultimately killed by the 500 mg/m<sup>2</sup> dosage in tunnels (85%) was considerably higher than that recorded against the same strain (VKPER) in tunnel tests with field rates of permethrin 500 mg/m<sup>2</sup> (30%) or deltamethrin 25 mg/m<sup>2</sup> (38%) (Hougard et al. 2003, Corbel et al. 2004a). A dosage of 500 mg/m<sup>2</sup> would be an appropriate field rate to evaluate on nets in experimental huts in phase II trials.

Rapid action is a desirable attribute for insecticides used in domestic situations. In this respect, the re-



**Fig. 2.** Efficacy of indoxacarb and permethrin treated netting against *An. gambiae* in tunnel tests. (A) Delayed mortality to indoxacarb over 24 h to 120 h (VKPER strain). (B) Dosage-mortality response of VKPER and Kisumu strains to permethrin and indoxacarb. Mortality was scored after 24 h in permethrin tests and after 96 h in indoxacarb tests. (C) Proportion penetrating the holed indoxacarb-treated netting and proportion of blood feeding among those that penetrated (VKPER). (D) Proportion penetrating the holed permethrin-treated netting and proportion blood feeding among those that penetrated (VKPER). (E) Indoxacarb tests on VKPER: proportion blood feeding and proportion dead after 48 h among those that blood fed. (F) Permethrin tests on VKPER: proportion blood feeding and proportion dead after 48 h among those that blood fed.

sponse of indoxacarb was disappointing. Repellent activity is prerequisite for achieving good personal protection. Indoxacarb failed on that score, too. Although the excitorepellency response to pyrethroids tends to lessen in mosquitoes that are resistant to this class of insecticide, the time to first take-off, as re-

ported by Hougard et al. (2003), was still several times shorter with permethrin (13 s) and deltamethrin (29 s) than with indoxacarb (98 s) against VKPER. It is the twin characteristics of fast knockdown and high irritancy that explain why pyrethroids were an ideal class of insecticide to use on nets for individual protection

**Table 3.** Tests for synergy on netting treated with indoxacarb, deltamethrin, and mixtures of the two insecticides

Deltamethrin concn (mg/m <sup>2</sup> )	% mortality (no. tested)	Indoxacarb concn (mg/m <sup>2</sup> )	% mortality (no. tested)	Deltamethrin/indoxacarb mixture	% mortality (no. tested)	Expected mortality	P
0.626	47 (30)	200	24 (29)	0.625/200	58 (31)	60	0.774
1.25	53 (30)	200	24 (29)	1.25/200	73 (26)	62	0.098
2.5	81 (32)	500	87 (31)	2.5/500	100 (32)	98	0.156



before the development of resistance. Despite these deficiencies, if indoxacarb-treated nets were to attain high coverage in a community, rather than providing individual protection through excitorepellency, the nets should give community protection and reduce transmission through a mass killing effect of the mosquito population. Applied in this way indoxacarb's twin characteristics of nonrepellency, so mosquitoes stay in contact long enough to pick up a lethal dose, and lack of cross-resistance to problematic resistance mechanisms would be positive attributes. Were indoxacarb treated nets to be distributed for free to entire communities, they could have a major impact on pyrethroid-resistant vector populations. If instead indoxacarb-treated nets were sold incrementally (e.g., through social marketing), they would fail to provide at low coverage levels the personal protection expected by individual users. The free net and social marketing approaches to scaling up of ITN each has their advocates and detractors (e.g., Curtis et al. 2003, Lines et al. 2003). Some argue that free nets is the only satisfactory approach to achieve high coverage or mass killing of mosquito populations necessary to protect everyone (Maxwell et al. 2002, Hawley et al. 2003), whereas others argue that private sector or non-governmental organisation-driven systems are the more feasible or sustainable approach to increasing coverage (Abdulla et al. 2001, Lines et al. 2003). We do not take a position in the debate here but point out that a net treated with two complementary insecticides might serve both systems equally well: the pyrethroid component to provide repellency and protection to individual users, and the indoxacarb to provide the mosquito-killing effect and community protection. Thus, we tested a net treated with a pyrethroid-indoxacarb combination. We hoped the mixture might prove synergistic. No synergism was observed, but the crucial finding was the absence of any antagonism between the two insecticides, so development of a combination formulation continues to hold promise.

The inherent characteristics of nonrepellency and slow activity are shared by indoxacarb and the cyclo-diene insecticides once used successfully for indoor residual spraying in the 1950s and 1960s (Brown and Pal 1971). Indoxacarb might therefore be usefully deployed as IRS if formulations were to be developed that showed adequate residual activity on interior walls. A slow mode of action is not necessarily an obstacle to transmission control because mosquitoes must survive several days (the length of the sporogonic cycle) before they can transmit an acquired infection. IRS trials of appropriate formulations are therefore warranted.

Aside from anophelines, ITN also are used as a barrier against biting by *Culex quinquefasciatus* Say, a nuisance mosquito and filariasis vector that has become difficult to control owing to complex patterns of resistance involving *kdr* (Chandre et al. 1998), elevated esterases and insensitive acetylcholinesterases (Chandre et al. 1997). Because indoxacarb is not affected by *kdr* or *Ace-1<sup>R</sup>* and is bioactivated by esterases to its more toxic metabolite DCJW (Wing et al. 2000,

Lapied et al. 2001) indoxacarb treated nets might show superior effectiveness against *Cx. quinquefasciatus*, which would contribute to their popularity.

### Acknowledgments

We thank Hounkarin Lazare for rearing of mosquitoes and Bill McClellan (DuPont) for providing the indoxacarb formulations. This work was supported by the Gates Malaria Partnership.

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Received 11 June 2006; accepted 20 November 2006.