Repellent and Deterrent Effects of SS220, Picaridin, and Deet Suppress Human Blood Feeding by *Aedes aegypti*, *Anopheles stephensi*, and *Phlebotomus papatasi*

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ABSTRACT A series of behavioral tests with *Aedes aegypti* (L.), *Anopheles stephensi* Liston, mosquitoes, and the sand fly *Phlebotomus papatasi* Scopoli in the presence of Deet, SS220, and Picaridin topically applied to the skin of human volunteers showed that the insects were deterred from feeding on and repelled from surfaces emanating the compounds. When offered a 12- or 24-cm^2 area of skin, one-half treated with compound and one-half untreated, the insects fed almost exclusively on untreated skin. The sand flies and mosquitoes did not at any time physically contact chemically treated surfaces. When treated and untreated skin areas were covered with cloth, insects contacted, landed, and bit only through cloth covering untreated skin. These observations provided evidence that the compounds deterred feeding and repelled insects from treated surfaces primarily as a result of olfactory sensing. When cloth, one-half untreated and one-half treated with chemical, was placed over untreated skin, insects only touched and specifically bit through the untreated cloth. This showed that the activity of the chemicals does not involve a chemical × skin interaction. In the presence of any of the three chemicals, no matter how they were presented to the insects, overall population biting activity was reduced by about one-half relative to controls. This reduction showed a true repellent effect for the compounds. Results clearly showed that Deet, SS220, and Picaridin exert repellent and deterrent effects upon the behavior of mosquitoes and sand flies. Heretofore, the combined behavioral effects of these compounds upon mosquito and sand fly behavior were unknown. Moreover, protection afforded by Deet, SS220, and Picaridin against the feeding of these three disease vectors on humans is mechanistically a consequence of the two chemical effects.

KEY WORDS N,N-diethyl-3-methylbenzamide, (1S, 2′S)-methylpiperidinyl-3-cyclohexene-1-carboxamide, 2-(2-hydroxyethyl)-1-piperidine carboxylic acid 1-methylpropyl ester, malaria vector, yellowfever mosquito

IT IS KNOWN THAT DEET (*N*,*N*-diethyl-3-methylbenzamide), SS220 [(1*S*,2′*S*)-methylpiperidinyl-3-cyclohexene-1-carboxamide], and Picaridin [2-(2-hydroxyethyl)-1-piperidine carboxylic acid 1-methylpropyl ester] offer protection against the bites of blood-feeding arthropods that vector human disease (Klun et al. 2003, Frances et al. 2004, Carroll et al. 2005). Despite the widespread knowledge of the protective qualities of these compounds, as measured by reduced bites sustained in field or laboratory tests, there is little information available on how the compounds mechanistically affect whole-organism behavior and thereby suppress the biting of disease vectors. Information in this area is particularly scarce for SS220 and Picaridin. However, for Deet, which was discovered decades before SS220 and Picaridin (Gilbert et al. 1955), there is a slightly larger bank of information. Schreck et al. (1970) studied the action of Deet and candidate mosquito repellents. They observed that, "mosquitoes exposed to an arm treated with repellent approach but do not land, land momentarily, land and walk, probe, and bite, in that order, as the amount of repellent is reduced, either artificially or by aging." Therefore, repellents do have a spatial effect that contributes to preventing insect attack." They went on to say, "none of the repellents completely prevented all mosquitoes from responding to the test subjects (humans). Therefore, repellents may initially reduce annoyance by preventing close approach of a relatively high proportion of avid mosquitoes, but complete protection from biting depends on both spatial and contact repellency." It should be noted, however, that Schreck et al. (1970) did not present any behav-

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ioral evidence to support the existence of a so-called contact repellency effect.

Boeckh et al. (1996) studied the protective efficacy of Deet and Picaridin against yellowfever mosquito, *Aedes aegypti* (L.); *Culex quinquefasciatus* Say; *Anopheles stephensi* Liston; and *Stomoxys calcitrans* (L.) and showed that Picaridin protected guinea pigs from bites for longer times, postapplication, than Deet. Using a Y-tube olfactometer with *Ae. aegypti*, they also found that both compounds equivalently reduced, but did not eliminate, the approach of the mosquitoes in response to host odor. Using an indirect molecular genetics approach to understand the effects of Deet on insect behavior, Reeder et al. (2001) isolated a mutant of *Drosophila melanogaster* Meigen that was not repelled by Deet and compared it with flies of a wild type that were highly repelled in an olfactory response assay. They concluded that Deet repellent effect in fruit flies was because of its airborne vapors. Hoffmann and Miller (2002, 2003) also showed that Deet has an olfactory effect. Deet evaporated upwind of attractant-baited traps or human subjects reduced mosquito upwind orientation to an attractive source compared with moving air without the compound. Carroll et al. (2005) showed that the repellent activity of Deet and SS220 against ticks involved olfactory sensing. We report the results of a series of bioassays with *Ae. aegypti*, the malaria vector *An. stephensi*, and the leishmaniasis vector, *Phlebotomus papatasi* Scopoli, which show that Deet, SS220, and Picaridin exert two behavioral effects on the insects. One is a feeding deterrent effect and the other is a repellent effect. Both effects are expressed as result of insect olfactory perception of the compounds.

Materials and Methods

Chemicals. Deet was obtained from Morflex, Inc. (Greensboro, NC) and Picaridin was from Bayer Consumer Care (Morristown, NJ). The compounds were at least 98% pure chemically according to gas chromatographic analyses. SS220 was synthesized previously at the Chemical Affecting Insect Behavior Laboratory (Beltsville, MD), and it was of 95% stereoisomeric and >99% chemical purity (Klun et al. 2003).

Bioassays. The bioassays involved controlled exposure of two 25–30-yr-old females and five 26–63-yr-old male Caucasian volunteers to feeding mosquitoes and sand flies. Each bioassay used a minimum of three individuals and at least one within the group was involved in one or more other bioassay tests. We adhered to the guidelines established by the National Institutes of Health for tests involving humans, and protocols were approved by the Human-Use Review Board of the Walter Reed Army Institute of Research (WRAIR) (Silver Spring, MD). SS220, Deet, and Picaridin had abundant safety databases (Klun et al. 2003) that permitted applications to the volunteers. All bioassays were conducted by using "K & D modules" and methods described by Klun and Debboun (2000). The bioassays used rectangular Plexiglas modules (26 by 5 by 5 cm) containing six isolated cells with a 3 by 4-cm trapdoor opening in each cell. Insects held in individual cells were exposed to the surfaces (skin or cloth) below the trap door with one-half of the surface treated with chemical and the other half untreated. In one case, we used 26 by 5 by 5-cm modules having four isolated cells with 4 by 6 cm trap door openings, to compare responses of mosquitoes exposed to 24- and 12-cm^2 half chemical-treated and half untreated skin areas. The half-cell bioassay design resembled a design used by Salafsky et al. (2000) to evaluate the attachment responses of ticks contained in screened cups over rabbit ear skin half treated with Deet and half untreated.

In all tests, adjacent cells of the modules were each fitted with five flies, the insect-charged modules were placed over the thigh of a human volunteer to which specific chemical treatments were applied, and doors of the cells were opened to expose the insects to the treatments below. The bioassays were replicated. Details of the bioassay tests are presented as follows.

Insects. *Ae. aegypti* (red eye Liverpool strain) *An. stephensi*, and *P. papatasi* used in the study were from pathogen-free colonies maintained at the WRAIR. The mosquitoes were reared (Gerberg et al. 1994) by feeding larvae ground tropical fish flakes (Tetramin Tropical Fish Flakes, Tetra Sales, Blacksburg, VA; www.tetra-fish.com). Adults were maintained in a photoperiod of 12:12 (L:D) h (with lights on at 0600 hours) at 27°C and 80% RH with cotton pad moistened with 10% aqueous sucrose solution. Mated nulliparous *Ae. aegypti* and *An. stephensi* females (5–15 d old) were tested. *An. stephensi* had access to water 24 h before testing and *Ae. aegypti* had no water 24 h before testing. *P. papatasi* was reared using methods described by Modi and Rowton (1999). The nulliparous females were 1–3 d old before being used in the bioassay tests. All tests with each species of mosquitoes and sand flies were done in a walk-in incubator (27°C and 80% RH) in ambient fluorescent light from 0800 to 1030 hours over 1 or 2 d. The insects were destroyed by freezing after being used once in a test.

Organdy Cloth. The fabric (G-Street Fabrics, Rockville, MD) was a tightly woven blend of 70% polyester and 30% nylon fibers. Optical-micrometer measurement of the 0.1-mm-thick woven cloth surface showed that it consisted of alternating 0.04- and 0.07-mm^2-sized holes. The cloth was used to interfere with insect contact with chemically treated and untreated skin surfaces, but it allowed vapor-phase penetration of chemicals and permitted the insects to bite through it. The cloth also was treated with chemicals and positioned over untreated skin of volunteers.

Four tests, each involving 60, 90, or 120 insects of each species, were conducted as follows. In all tests, the surface contact and biting behavior of flies on chemically treated and untreated surfaces were recorded.

Test 1. Compounds on Skin. Volunteers wearing short pants were seated. Using a skin-marking template and a washable-ink marker, skin areas representing 3 cm by 4-cm floor openings of four cells of the

K & D module were outlined on the outer, top, and inner thigh positions of each leg. Each set of the four 12-cm^2 rectangular skin areas was divided with a midline mark. One half of the skin in each rectangle (6 cm^2) was randomly treated with 27.5 μl of ethanol alone (control) or 27.5 μl of ethanol containing 288 nmol of SS220, Picaridin, or Deet by using a pipette to yield 48 nmol of compound/cm^2 on 6-cm^2 area of skin. This dose, for all compounds, was previously determined to cause an 80% reduction in *An. stephensi* and *Ae. aegypti* biting on humans compared with untreated skin (Klun et al. 2003), and it was considered an appropriate dose for the behavioral tests. The four treated cell rectangles each represented a randomized block, and each volunteer had three blocks on each of two thighs. In practice, treatments were applied to two blocks at a time, and after the treated skin dried completely of solvent, it was exposed to the insects. Compounds were applied to the next two blocks after tests with the first two blocks were complete. This sequence was repeated until six blocks on each volunteer had been exposed to the insects. To expose insects to the treatments, four cells of a K & D module were each fitted with five insects, the module was positioned over treated skin areas, and the trap doors of the cells were opened to expose the insects to the skin below. The number of insects that contacted untreated or treated skin, and the number biting on the respective surfaces in a 2-min exposure period were observed and recorded. Insects were prodded back into the module cells, and the trapdoors were closed to conclude the test. Biting insects were those that became engorged with blood or had their mouthparts inserted into volunteers' skin within the 2-min exposure period.

Test 2. Skin Surfaces (12 and 24 cm^2) Half Chemically Treated and Half Untreated. This test was conducted similarly to test 1. However, in test 2, we evaluated the response of *Ae. aegypti* exposed to 12- and 24-cm^2 skin areas when half of the respective areas were treated with the chemical. Three sets of skin areas representing 3 by 4-cm floor openings of four cells of the standard K & D module were outlined with washable ink on the outer, top, and inner thigh positions of one of a volunteer's leg. Three sets of skin areas representing four 4 by 6-cm trapdoor floor opening of a larger four cell K & D type module were outlined on the outer, top, and inner thigh positions of the volunteer's other leg. The skin areas on the respective legs were divided with a midline mark. One-half of the skin in a 12-cm^2 rectangular area was treated with 27.5 μl of ethanol alone (control) or 27.5 μl of ethanol containing 288 nmol of SS220, Picaridin, or Deet by using an automatic pipette. The other half-area of the skin was untreated. Similarly, one-half of the skin in each 24-cm^2 rectangular area was treated with 55 μl of ethanol alone (control) or 55 μl of ethanol containing 576 nmol of SS220, Picaridin, or Deet by using an automatic pipette. Thus, in both cases, the dose of the compound applied to the skin was 48 nmol/cm^2. Four adjacent cells of the respective sized K & D modules were each filled with five *Ae. aegypti*, the modules were positioned over the treatments, and the exposed areas to the skin and the mosquito responses were recorded as described in test 1.

Test 3. Compound-Treated Skin Covered with Cloth. Half-cell skin areas were treated with ethanol (control), SS220, Picaridin, and Deet as described in test 1. A 7 by 30-cm length of organdy cloth, the size of the K & D module base, was placed over a block of treatments, and then four cells of a K & D module each filled with five insects were positioned over the cloth-covered treatments. Trapdoors of the module were opened, and the insects were exposed to the cloth. The purpose of the cloth was to interfere with direct insect contact with skin. After a 2-min exposure, the number and position of insects that touched and bit through the cloth were recorded, and the doors of the module were closed to conclude the test.

Test 4. Compound-Treated Cloth Covering Untreated Skin. Three sets of four 12-cm^2 rectangular areas were marked on left and right thighs of volunteers as described in test 1. Complementary 3 by 4-cm areas were traced onto 7 by 30-cm lengths of cloth. Each of the four 12-cm^2 rectangular cloth areas within a block were divided by a midline-mark, and 6 cm^2 of the cloth in each rectangle was randomly treated with 27.5 μl of ethanol alone (control) or 27.5 μl of ethanol containing 288 nmol of SS220, Picaridin, or Deet. The cloth was held in a chemical fume hood until the solvent evaporated to dryness. The cloth was positioned over the volunteers' untreated skin $\approx$15 min after drying, and insects in the K & D module were exposed to the treatments. The exposure lasted for 2 min, and as stated previously, the insect responses to treated and untreated surfaces were recorded.

SE values for the percentage insects biting on compound-treated or untreated surfaces were calculated: $SE = \sqrt{p(1-p)/n} \times 100$, where p is the proportion of insects biting and n is number of insects observed to calculate p.

Results and Discussion

Dethier et al. (1960) discussed the importance of the terminology used to describe the behavioral effects that chemicals elicit from insects. They defined five standard terms (arrestant, stimulant, attractant, repellent, and deterrent) to describe chemicals in terms of the responses they evoke, and it was proposed that the terms be used as standards within the limits of the definitions. Dethier et al. (1960) emphasized further that there was significant value in using the standard terms because they permitted unambiguous description of the behavioral effects that chemicals evoke. They also contended that it was of more than academic interest to use the precise terminology to avoid confusion and to nurture a greater mutual understanding among contemporary behaviorists on how chemicals affect insect behavior. Among the five standard terms, Dethier et al. (1960) defined a repellent as a chemical that causes insects to make oriented movement away from its source, and a deterrent as a chemical that inhibits feeding or oviposition when

Table 1. Biting percent of mosquitoes and sand flies on adjacent treated and untreated half-cell surfaces with (*n*) number of mosquitoes and sand flies tested against each treatment (test 1)

Insect/biting site	% insects biting (SE)				*n*
	Control	SS220	Picaridin	Deet	
Ae. aegypti					
Treated skin	36.6	0.0	2.6 (1.4)	2.6 (1.4)	120
Untreated skin	35.0	31.6 (4.2)	43.4 (4.8)	30.8 (4.2)	
Total	71.6 (4.1)				
An. stephensi					
Treated skin	28.8	0.0	0.0	0.0	90
Untreated skin	35.0	31.6 (4.9)	43.4 (5.2)	30.8 (4.8)	
Total	63.8 (5.1)				
P. papatasi					
Treated skin	23.2	0.0	0.0	0.0	90
Untreated skin	47.8	58.8 (5.2)	50.0 (5.3)	43.4 (5.2)	
Total	71.0 (4.8)				

Treatments of SS220, Picaridin, and Deet were each at a dose of 48 nmol of compound/cm^2.

present in a place where insects would, in its absence, feed or oviposit. We use these definitions in the discussion of our study.

Tables 1–4 show the results of the bioassays providing evidence that SS220, Picaridin, and Deet all exerted both deterrent and repellent effects on the insects. The half-cell treatments showed that among feeding insects, 98 to 100% of bites occurred on untreated skin (Tables 1 and 2), on untreated skin covered with cloth (Table 3), or through untreated cloth covering untreated skin (Table 4). In repeated observations of responses of insects in cells simultaneously exposed to half-chemically treated and -untreated surfaces, we observed that insects never made physical contact with treated surfaces in the cells, and always flew down and bit on or through untreated surfaces. These observations revealed that the mosquitoes and sand flies detected a vapor-phase olfactory gradient of chemical within the K & D module cells in the 2-min exposure period and fed, almost entirely, upon untreated surfaces in cells containing the compounds. Thus, our results definitely show that SS220, Picaridin, and Deet are deterrents because, according to the definition, they inhibited feeding when present in a place where the insects would normally feed if the chemicals were absent.

Table 2. Percentage of *Ae. aegypti* biting on adjacent treated and untreated half-cell 12- and 24-cm^2 skin surfaces with (*n*) number of mosquitoes tested against each treatment (test 2)

Skin area/biting site	% insects biting (SE)				*n*
	Control	SS220	Picaridin	Deet	
24 cm^2					
12 cm^2, treated skin	35.0	0.0	1.6	0	60
12 cm^2, untreated skin	40.0	23.0 (5.4)	35 (4.5)	25 (5.6)	
Total	75.0 (5.0)				
12 cm^2					
6 cm^2, treated skin	41.7	0.0	0.0	1.6	60
6 cm^2, untreated skin	35.0	20.0 (5.2)	41.7 (6.4)	27.0 (5.7)	
Total	76.7 (5.5)				

Treatments of SS220, Picaridin, and Deet were each at a dose of 48 nmol of compound/cm^2 skin.

Tables 1, 3, and 4 show that among all mosquitoes and sand flies approximately twice as many of them consistently bit in control cells than in cells containing compound treatments, and biting in the controls cells was generally distributed equally across blank solvent-treated and untreated sides of the cells. For example, test 1 data with *Ae. aegypti*, show that a total of 71.6% of 120 mosquitoes bit in the control cell, and the bites were distributed nearly equally; 36.6% on solvent-treated skin and 35% on untreated skin (Table 1). In contrast with the 71.6% total biting in the control, a total of only 31.6, 46, and 33.4% of all mosquitoes bit on untreated skin in cells containing SS220, Picaridin, and Deet, respectively. Moreover, it was very striking that the percentage of mosquitoes biting on untreated areas was very similar to the percentage of biting on each skin side in control cells. This phenomenon was observed repeatedly throughout the tests with each species. Test 2 results with *Ae. aegypti* confirmed the 50% overall bite reduction in cells containing chemicals compared with control cells that was observed in test 1 (Table 2). Test 2 results also demonstrated that 50% bite reduction was independent of the total area of skin that was available for feeding. Moreover, data showed that volatiles associated with chemically treated skin not only prevented feeding on the treated skin but also suppressed feeding on adjacent untreated skin, and the suppression was independent of the area of attractive skin available for feeding. We think that the bite reduction is the result of olfactory-based orientated movement of the insects away from the source of compound in the cells, and this shows repellent effects of SS220, Picaridin, and Deet. It is also remarkable that the 50% overall population biting reduction was about the same for all three species of insects. We hypothesize that a dose by response relationship exists for this repellent effect and doses higher than 48 nmol of compound/cm^2 can evoke levels of bite reduction >50%. The repellent effect we observed for SS220, Deet, and Picaridin is consistent with the repellent effect of the various compounds observed by Schreck et al. (1970), Boeckh et al. (1996), Hoffmann and Miller (2003), and Carroll et al. (2005) in arthropods.

In test 3, chemically treated skin and untreated skin areas in modules cells were covered with organdy cloth (Table 3). The data showed that despite the fabric covering, the insects preferentially bit through the cloth covering untreated skin. As was the case in other tests, we observed that these biting flies never physically contacted cloth covering treated skin. These results provided additional evidence that the insects detected the location of compounds on the skin by olfactory sensing.

Table 4 shows results of test 4. The data showed that the three species avoided areas of SS220- and Picaridin-treated cloth and bit through untreated cloth surfaces to the untreated skin below. The results demonstrated that the deterrent and repellent effects of the chemicals did not involve a skin by compound interaction and indicated that all three compounds could offer protection against biting if they were ap-

Table 3. Percent biting on adjacent treated and half-cell skin surfaces covered with cloth (test 3)

Insect/biting site	% insects biting (SE)				*n*
	Control	SS220	Picaridin	Deet	
Ae. aegypti					
Treated skin					
Cloth covered	31.2	6.6 (2.6)	12.8 (3.5)	8.8 (2.9)	90
Untreated skin					
Cloth covered	60.0	50.0 (5.3)	67.8 (4.9)	52.2 (5.3)	
Total	91.2 (3.0)				
An. stephensi					
Treated skin					
Cloth covered	25.6	1.2 (1.1)	1.2 (1.1)	0.0	90
Untreated skin					
Cloth covered	38.8	47.8 (5.3)	41.2 (5.2)	27.8 (4.7)	
Total	64.4 (5.0)				
P. papatasi					
Treated skin					
Cloth covered	36.6	0.0	0.0	0.0	90
Untreated skin					
Cloth covered	34.4	37.8 (5.1)	37.8 (5.1)	28.8 (4.8)	
Total	71.0 (4.7)				

Number of mosquitoes and sand flies tested against each treatment (*n*). Treatments of SS220, Picarid, and Deet were each at a dose of 48 nmol of compound/cm^2.

plied to clothing. This is in agreement with numerous previous studies that showed Deet-treated clothing can offer protection against ticks and biting flies (Grothaus et al. 1976; Mount and Snoddy 1983; Schreck et al. 1979, 1986; Evans et al. 1990).

Test 4 showed that Deet applied to cloth failed to deter the biting of *P. papatasi*, whereas SS220 and Picaridin were highly effective against it. Initially, we speculated that the loss of activity might be because of entrainment of the Deet in the cloth owing to its known solubility in plastics, and a concomitant reduction in the amount of Deet in the vapor phase, which permitted break-through biting by the sand fly. Subsequent study of this phenomenon, however, revealed that the break-through biting with Deet-treated cloth was because of a differential rate of evaporation between Deet and the other two compounds. Previously published data showed that in time-course studies, Deet evaporated more rapidly from organdy cloth than SS220 or Picaridin and that the residual Deet present was insufficient to deter sand fly biting.

Table 4. Percent biting on adjacent treated and untreated half-cell cloth surfaces with (*n*) number of mosquitoes and sand flies tested against each compound (test 4)

Insect/biting site	% insects biting (SE)				*n*
	Control	SS220	Picaridin	Deet	
Ae. aegypti					
Treated cloth cover					
Untreated skin	46.6	1.2 (1.1)	1.2 (1.1)	7.8 (2.8)	90
Untreated cloth cover					
Untreated skin	43.4	40.0 (5.1)	46.6 (5.2)	38.8 (5.1)	
Total	90.0 (3.1)				
An. stephensi					
Treated cloth cover					
Untreated skin	21.6	1.6 (1.3)	0.0	0.0	60
Untreated cloth cover					
Untreated skin	25.0	26.6 (4.6)	21.6 (4.3)	23.4 (4.5)	
Total	46.6 (6.4)				
P. papatasi					
Treated cloth cover					
Untreated skin	26.6	5.0 (2.8)	0.0	28.4 (5.8)	60
Untreated cloth cover					
Untreated skin	35.0	46.6 (6.4)	46.6 (6.4)	31.6 (6.0)	
Total	61.6 (6.2)				

Treatments of SS220, Picaridin, and Deet were each at a dose of 48 nmol of compound/cm^2.

Overall, our study proved that SS220, Picaridin, and Deet each exerted olfactory-based repellent and feeding deterrent effects upon three important vectors of human diseases. The combination of these effects probably accounts for the personal protection properties of the compounds. Deet and similar behaviorally active chemicals are sold widely as "insect repellents." This is a loosely accurate semantic reality that will probably never change. However, it is imperative that terminology used in studies of insect behavior should more accurately describe such chemicals in terms of the actual effects they exert on insect behavior. This accuracy is of importance because research progress toward development of new behaviorally active chemical tools for protection of humans against disease vectors will ultimately depend upon the extent to which the fundamental nature of the processes that influence insect behavior are accurately described and understood.

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