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LABORATORY EVALUATION OF AI3-37220, AI3-35765, CIC-4, AND DEET REPELLENTS AGAINST THREE SPECIES OF MOSQUITOES[1]

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ABSTRACT. Four repellents, *N,N*-diethyl-3-methyl-benzamide (deet), 2-hydroxy-methyl-cyclohexyl acetic acid lactone (CIC-4), and 2 piperidines (1-[3-cyclohexen-1-ylcarbonyl] piperidine [AI3-35765] and 1-[3-cyclohexen-1-ylcarbonyl]-2-methylpiperidine [AI3-37220]) were evaluated alone and in combination against *Aedes aegypti, Anopheles stephensi,* and *Culex quinquefasciatus* using a modified in vitro test system. This method was a valuable tool for comparing effective concentrations of the new compounds. Because of the controlled conditions of the test, it was possible to use the results of assays that had been conducted over a 5-year period and to perform the many replications necessary to evaluate combinations of compounds. The new candidate repellents were generally as effective as deet. Although speculative at this time, there was some evidence of synergistic interaction. Repellent combinations of CIC-4/AI3-37220/AI3-35767, deet/AI3-35765, and deet/AI3-37220/AI3-35765 against *An. stephensi* and CIC-4/AI3-35765, deet/AI3-37220/AI3-35765, AI3-37220/AI3-35765, and CIC-4/AI3-37220 against *Ae. aegypti* were more effective than the component compounds alone.

KEY WORDS *Aedes aegypti, Anopheles stephensi, Culex quinquefasciatus,* piperidines, lactone, in vitro

INTRODUCTION

New arthropod repellents are needed for improved efficacy against a wide range of biting arthropods. In contrast to vaccines and chemoprophylaxis as means of personal protection, use of repellents has the advantage of offering protection against a broad range of arthropod-borne diseases (Webster et al. 1991, Gupta and Rutledge 1994). The Walter Reed Army Institute of Research (WRAIR) Arthropod Repellent Program is directed toward finding an improved arthropod repellent formulation that provides: 1) protection equal to or better than that of the current military insect repellent, a multipolymer sustained-release formulation of *N,N*-diethyl-3-methylbenzamide (deet); 2) prevents bites for 12 or more hours under a variety of environmental conditions; 3) is safe to use; and 4) is acceptable to the user and pleasant to apply on the skin. Although deet is currently the most widely used repellent and is arguably one of the most successful products to improve public health (Osimitz and Grothaus 1995), it has certain drawbacks. Deet is not completely effective against some insects (Rutledge et al. 1985, Schreck 1985, Buescher et al. 1987, Curtis et al. 1987). Also, despite numerous safe applications of deet, there are concerns about its potential toxicity and safety (Ambrose 1959, Gryboski et al. 1961, Robbins and Cherniack 1986, Moody 1989, Lipscomb et al. 1992, Veltri et al. 1994) and possible health risks associated with repeated use at high concentrations (Oransky et al. 1989).

Improvements would be desirable in increased persistence on human skin (Mehr et al. 1985), reduced absorption (Gupta and Rutledge 1989, Gupta et al. 1990), user acceptability by American soldiers (Hooper and Wirtz 1983, Gambel 1995), and compatibility with military materiel.

Previous studies have reported the laboratory and field efficacy of several new repellent compounds individually: the lactone, 2-hydroxy-methyl-cyclohexyl acetic acid lactone (CIC-4), and the piperidine compounds 1-(3-cyclohexen-1-ylcarbonyl)-2-methylpiperidine (AI3-37220) and 1-(3-cyclohexen-1-ylcarbonyl) piperidine (AI3-35765) (Schreck and McGovern 1989; Robert et al. 1992; Coleman et al. 1993, 1994; Perich et al. 1995; Solberg et al. 1995; Frances et al. 1996; Walker et al. 1996). This in vitro study was initiated in 1991 and continued into 1996 to evaluate the feasibility of combining 2 or more repellent compounds for repellency against a broad range of medically important arthropods, to determine which of the compounds would enhance the standard Department of Defense (DOD) repellent containing deet, and the possibility that 1 of these compounds or a combination of them without deet could replace the current DOD repellent.

MATERIALS AND METHODS

Test insects: Laboratory-reared female mosquitoes used in this study were *Aedes aegypti* (Linn.)

[1] The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the U.S. Department of the Army. Use of trade names does not constitute an official endorsement or approval of the products mentioned.

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(Red eye Liverpool strain), *Anopheles stephensi* Liston (Delhi strain, originally colonized from India), and *Culex quinquefasciatus* Say. Mosquitoes were reared and maintained in the WRAIR Insectary at the Department of Entomology using procedures as described by Hoch et al. (1995). Mosquitoes used for experimentation were nulliparous females between 5 and 15 days old. Mosquitoes were tested at 27°C ambient air temperature and 78% relative humidity and were provided only water for 24 h before testing.

Test repellents: The following technical-grade chemicals were tested: deet (Sigma Chemical Company, St. Louis, MO); U.S. Department of Agriculture (USDA) piperidine compounds AI3-37220 and AI3-35765, synthesized by Terrence P. McGovern (Insect Chemical Ecology Laboratory, USDA, Beltsville Agricultural Research Center, Beltsville, MD); and CIC-4 (Angus Chemical Company, Northbrook, IL).

Repellent test procedure: A modified in vitro blood-feeding method as described by Rutledge et al. (1976) was used to evaluate the efficacy of repellents used alone and in combination against laboratory-reared mosquitoes. It incorporated an in vitro blood membrane feeder that allows unrestricted free choice feeding on the various repellent-treated membrane surfaces. The test system consisted of a membrane blood feeder attached to a constant-temperature water circulator to maintain the reservoir blood temperature at 37°C. The membrane blood feeder has 5 circular (3.0-cm-diameter) blood reservoirs (Fig. 1). The reservoirs were filled with 26 ml of outdated human blood obtained from the blood bank of the Walter Reed Army Medical Center, Washington, DC and supplemented with 72 mg of adenosine triphosphate (Rutledge et al. 1964). A 4.5-cm square of commercial baudruche (Joseph Long Inc., Belleville, NJ) was secured on the top of each reservoir with high vacuum grease (Dow Corning Corporation, Midland, MI). Absolute ethanol was used as a diluent for various repellent concentrations and also for the control reservoir. Combinations of repellents were prepared with equal concentrations of each component compound with the combined concentration of all compounds equal to the stated target concentration for the individual reservoir membrane. Repellents were applied at random to the 5 reservoirs using the following concentrations: 0.16, 0.08, 0.04, and 0.02 mg/cm^2. After 5 min, a test cage (30 × 30 × 30 cm) containing 250 female mosquitoes was placed over the set of 5 treated reservoirs. A slide door was withdrawn to expose the mosquitoes to all of the 5 reservoirs simultaneously. The number of mosquitoes feeding on the 4 treatment reservoirs and the control reservoir were counted at 2-min intervals for 20 min using the total number of probing mosquitoes as the response. Each test was replicated 4 to 16 times to obtain a statistically valid sample size for analysis. These tests were performed over a period of 5 years, taking advantage of our ability to perform the assays under controlled conditions. In this way, we were able to accumulate the large amount of data necessary to evaluate combinations of the repellents against the 3 vector mosquito species.

Statistical analysis: The percentage of mosquitoes feeding on each treated or control reservoir was transformed to the probit scale and repellent dosages were transformed into the logarithmic scale. Median effective doses to repel 50% (ED_{50}) of the mosquito test population were then calculated by the method of Goldstein (1964) for single curves with graded responses. Significant differences were determined by comparing the 95% confidence intervals among effective doses.

RESULTS AND DISCUSSION

Table 1 shows individual estimated ED_{50} values for deet, CIC-4, AI3-37220, and AI3-35765 obtained against the various mosquito species. During these in vitro studies, the average number of mosquitoes that fed on the control reservoir was 49.6 mosquitoes. Deet, CIC-4, AI3-37220, and AI3-35765 provided similar repellency against the biting mosquitoes. However, deet provided significantly better repellency ($P < 0.05$) than AI3-27220 against *Ae. aegypti.*

The ED_{50} values for deet, CIC-4, AI3-37220, and AI3-35765 when used in combination against laboratory-reared mosquitoes are presented in Table 2. Although there were no significant differences in repellency between deet and repellent combinations against *An. stephensi* and *Cx. quinquefasciatus,* there was a significant difference ($P < 0.05$) in repellency between deet and the repellent combination of deet/AI3-37220 against *Ae. aegypti.* There were significant differences ($P < 0.05$) observed in repellency between the repellent combinations of deet/AI3-35765 and CIC-4/AI3-37220 and between deet/AI3-35765 and deet/CIC-4/AI3-37220/AI3-35765 against *An. stephensi.* Although none of the differences between repellent combinations and deet showed statistically significant synergism, some combinations had a lower ED_{50} than deet (Table 2). These sorts of comparisons might eventually discover a useful repellent synergist. For example, the repellent combination of deet/AI3-35765 had a lower ED_{50} value than either compound used alone against *An. stephensi.* Similarly, the repellent combinations of CIC-4/AI3-35765, deet/CIC-4/AI3-37220/AI3-35765, AI3-37220/AI3-35765, and CIC-4/AI3-37220 each had a lower ED_{50} value than either compound used alone against *Ae. aegypti.* However, the apparent synergistic effect was not observed in other repellent combinations. In some cases, the candidate repellent compound exhibited greater repellency as compared to a combination of repellents. For example, AI3-37220 used alone against *Cx. quinquefasciatus* was more effective than when used in combination with any of the oth-

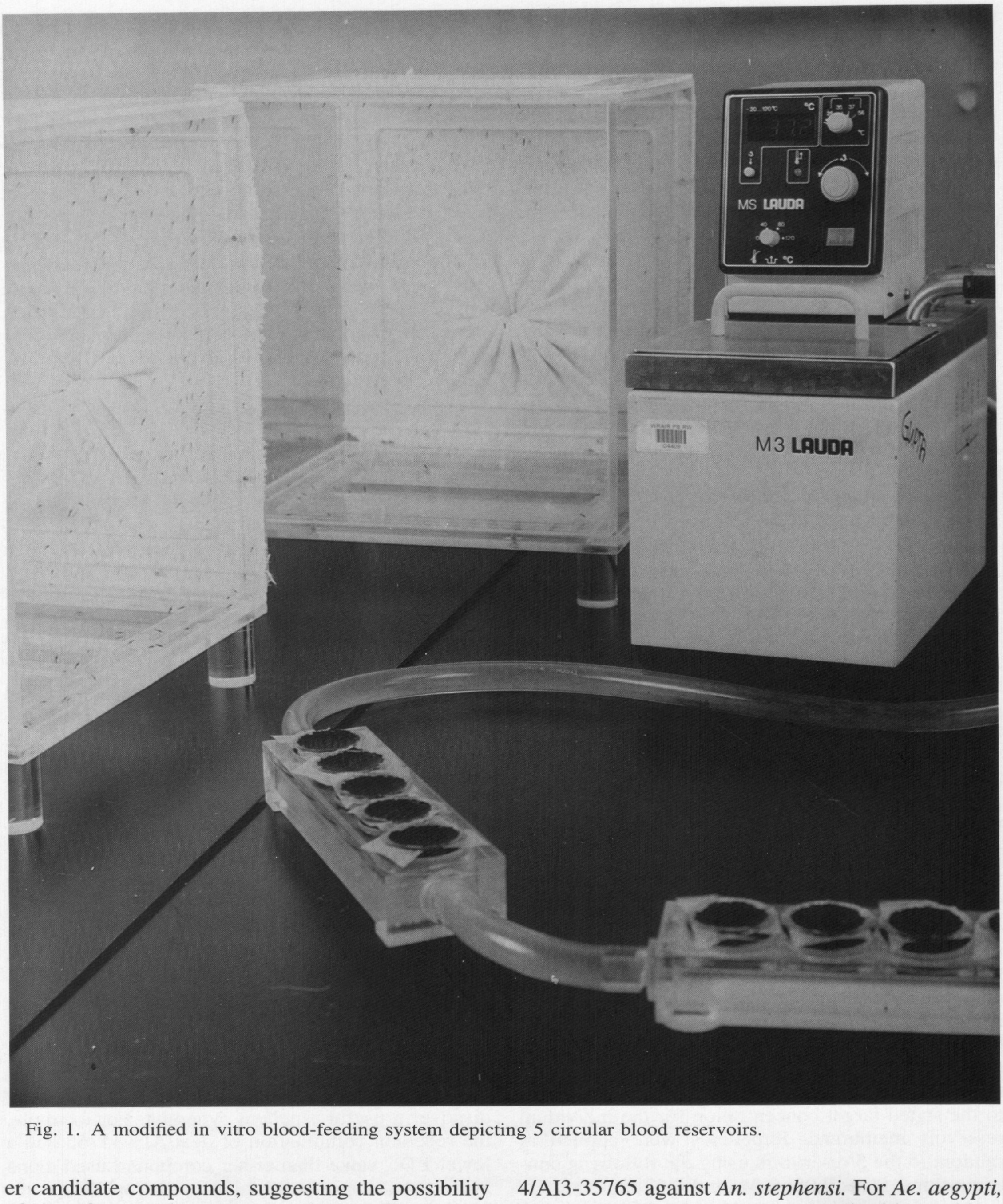

Fig. 1. A modified in vitro blood-feeding system depicting 5 circular blood reservoirs.

er candidate compounds, suggesting the possibility of interference between certain repellent compounds.

Some of the results also indicated that there was a great variation in slope of the log-dose probit line. Although the ED_{50} of CIC-4 against *An. stephensi* was low, the slope was very shallow, indicating that a high concentration of repellent would be required to repel a greater percentage of the mosquito population. The greatest slopes were associated with combinations of repellents CIC-4/AI3-37220/AI3-35765, deet/AI3-37220/AI3-35765, and deet/CIC-4/AI3-35765 against *An. stephensi.* For *Ae. aegypti,* the greatest slopes were associated with combinations of repellents AI3-37220/AI3-35765 and deet/AI3-37220. The increase in slopes of some combinations suggests an advantage of using combinations when a high level protection is required.

The purpose of this study was to determine the efficacy of CIC-4, AI3-37220, and AI3-35765 used alone or in various combinations against laboratory-reared *Ae. aegypti, An. stephensi,* and *Cx. quinquefasciatus* using an in vitro membrane blood-

Table 1. In vitro repellency of deet, CIC-4, AI3-37220, and AI3-35765 against various mosquito species.

Species	Repellent	Slope[1]	ED_{50} (μg/cm²) (95% CL)[2]
Anopheles stephensi	Deet	−3.4	29.5 (5.1–50.7)
	CIC-4	−1.9	4.8 (1.0–8.1)
	AI3-37220	−1.4	14.0 (0.1–14.3)
	AI3-35765	−3.1	29.4 (13.6–43.3)
Aedes aegypti	Deet	−1.4	13.3 (8.0–21.1)
	AI3-35756	−2.1	23.0 (0.0–49.7)
	CIC-4	−2.9	26.2 (16.7–75.1)
	AI3-37220	−1.9	50.6 (33.2–72.8)
Culex quinquefasciatus	Deet	−2.0	20.1 (9.1–29.9)
	AI3-37220	−1.4	13.2 (9.8–17.5)
	AI3-35765	−0.8	13.8 (9.6–19.8)
	CIC-4	−2.1	28.5 (13.5–41.8)

[1] Slope of log-dose probit regression line.
[2] ED_{50} values are significantly different ($P < 0.05$) from each other if 95% confidence limits (CL) do not overlap.

feeding system. Although results from this study demonstrated that deet, at the ED_{50} level, provided significantly better ($P < 0.05$) repellency than AI3-37220 against *Ae. aegypti,* in general, the 3 experimental compounds used alone showed similar repellency to deet (Table 1). The 3 experimental compounds in various combinations with deet or with each other showed similar repellency to deet except for the repellent combination of deet/AI3-37220 against *Ae. aegypti.* This study has provided for the 1st time, quantitative, comparative data indicating the possibility of synergism and interference. In vitro testing could be used to screen synergists as well as repellents (Table 2). Animal or field testing is always necessary to prove efficacy, but the in vitro technique is an inexpensive and efficient way to start. Repellents when used in combinations might increase effectiveness of a formulation against a wider variety of arthropod vectors, while exposing the user to the same or lower total exposure to topically applied chemicals. In addition, if a population of mosquitoes has a wide variation in response to a single repellent, use of a combination may offer the advantage of affecting those individuals with tolerance to any single compound in the mixture.

Table 2. In vitro repellency of repellent combinations against various mosquito species.

Species	Repellent	Slope[1]	ED_{50} (μg/cm²) (95% CL)[2]
Anopheles stephensi	Deet	−3.4	29.5 (5.1–50.7)
	CIC-4/AI3-37220/AI3-35765	−4.8	9.8 (0.0–20.4)
	Deet/AI3-35765	−2.3	14.5 (11.4–17.4)
	Deet/AI3-37220/AI3-35765	−4.6	16.3 (0.4–32.9)
	Deet/CIC-4/AI3-37220/AI3-35765	−3.0	28.1 (21.6–34.1)
	Deet/CIC-4/AI3-35765	−6.1	30.8 (6.8–51.9)
	Deet/AI3-37220	−3.3	36.7 (0.0–86.3)
	AI3-37220/AI3-35765	−2.4	39.9 (14.7–66.2)
	CIC-4/AI3-37220	−2.3	48.3 (31.0–69.3)
	CIC-4/AI3-35765	−4.2	49.9 (29.6–76.7)
Aedes aegypti	Deet	−1.4	13.3 (8.0–21.1)
	CIC-4/AI3-35765	−0.9	7.2 (0.5–16.4)
	Deet/AI3-37220/AI3-35765	−1.6	8.1 (0.0–22.9)
	AI3-37220/AI3-35765	−4.0	18.7 (0.3–37.2)
	CIC-4/AI3-37220	−1.6	21.4 (0.3–35.2)
	Deet/AI3-37220	−3.1	36.0 (24.9–46.7)
	Deet/CIC-4	−2.8	39.8 (18.6–61.7)
	Deet/AI3-35765	−2.4	42.8 (10.8–81.5)
Culex quinquefasciatus	Deet	−2.0	20.1 (9.1–29.9)
	AI3-37220/AI3-35765	−2.8	27.8 (15.1–39.1)
	CIC-4/AI3-37220	−3.7	36.2 (7.4–64.1)
	Deet/CIC-4	−3.3	40.3 (18.6–62.8)

[1] Slope of log-dose probit regression line.
[2] ED_{50} values are significantly different ($P < 0.05$) from each other if 95% confidence limits (CL) do not overlap.

Previous results evaluating these compounds alone using humans in laboratory and field studies demonstrated that their repellency was either less than, equal to, or better than deet against a variety of arthropods. For example, Schreck and McGovern (1985) reported that AI3-35765 was as effective or better than deet against *Mansonia titillans* (Walker). Robert et al. (1992) found that CIC-4 and AI3-37220 provided field and laboratory protection equal to or better than deet against the black flies *Prosimulium mixtum* Symes and Davies and *Prosimulium fuscum* Symes and Davies. Coleman et al. (1993) showed that repellencies of AI3-37220, AI3-35765, and CIC-4 were not markedly different from deet against laboratory-reared *Anopheles albimanus* Wiedemann, *Anopheles freeborni* Aitkin, *Anopheles gambiae* Giles, *An. stephensi,* and *Phlebotomus papatasi* (Scopoli). In 1994, Coleman et al. examined the relative efficacy of these compounds against laboratory-reared *Culex pipiens* L. using human volunteers and reported that CIC-4 was more effective than deet, AI3-37220, or AI3-35765 at the ED_{50} level; whereas at the ED_{95} level, deet provided significantly better protection than either piperidine compound. Perich et al. (1995) evaluated these compounds in the field against biting midges, *Leptoconops americanus* Carter and found that CIC-4 and AI3-35765 were significantly less effective than deet, whereas AI3-37220 was significantly more effective than deet. Solberg et al. (1995) evaluated deet and AI3-37220 on human volunteers against the lone star tick, *Amblyomma americanum* (L.) in the field and found that AI3-37220 showed significantly better repellency than deet. Frances et al. (1996) performed laboratory studies that demonstrated that *Anopheles dirus* Peyton and Harrison was more sensitive to CIC-4 than either AI3-37220 or deet; whereas, field studies showed that AI3-37220 provided significantly better protection against *An. dirus* than either deet or CIC-4. Walker et al. (1996) reported that AI3-37220 was more effective than deet in repelling *Anopheles arabiensis* Patton and *Anopheles funestus* Giles in western Kenya.

Our results are in agreement with other studies that indicate that AI3-35765 and AI3-37220 are as effective as deet (Shreck et al. 1978, 1979a, 1979b; Shreck and McGovern 1985; Robert et al. 1992; Coleman et al. 1993, 1994) and appear to be promising alternative candidates to deet when their broadspectrum repellent activity is considered. Therefore, further research with AI3-37220 and AI3-35765 is recommended.

Toxicological testing of mixtures of these compounds is necessary before combinations of the repellents can be evaluated using human volunteers. Only a mixture of deet and AI3-37220 has undergone toxicological testing (Snodgrass and Harvey 1995). Tested individually, these compounds presented no toxicological hazard (Weeks 1990, Haight et al. 1991, Griffin 1992, Angerhofer et al. 1996, Houpt and Snodgrass 1997). However, in some field trials, AI3-37220 and AI3-35765 induced a minor warming sensation on the skin but did not produce reddening or other visible signs. This sensation was only felt by a few susceptible individuals, particularly under hot, humid conditions (Coleman, Strickman, and Klein, personal communication). However, this response also varied from a slight warming to a minor heating sensation, similar to the "menthol effect."

This study showed that the repellents CIC-4, AI3-37220, and AI3-35765 used alone or in combination provided similar repellency compared to deet against laboratory-reared *Ae. aegypti, An. stephensi,* and *Cx. quinquefasciatus.* The in vitro tests provided a controlled assay to determine potential repellency of these compounds under controlled conditions, making it possible to observe interaction of compounds on repellency and to accomplish a large number of replicate trials.

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