Induction of Hyperactivity in Larvae of the Cattle Tick *Boophilus microplus* by Formamidines and Related Compounds

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The effect of chlordimeform and 18 related compounds on the aggregation behavior of the negatively geotactic larvae of the cattle tick, *Boophilus microplus* was investigated. Aggregations were treated and hyperactivity in the forms of immediate dispersal, delayed dispersal, and prolonged leg waving evaluated. A marked structure–activity relationship with delayed dispersal resulted; most active were the *N*-monomethyl formamidines, *N'*-(2,4-dimethylphenyl)-*N*-methylformamidine and demethylchlordimeform. Other compounds, including chlordimeform, with structures compatible with metabolism to *N*-monomethyl formamidines were also active. Delayed dispersal caused by those possessing the *N*,*N*-dimethyl moiety was antagonized after inhibition of oxidative *N*-demethylation to *N*-monomethyl analogs by piperonyl butoxide. Since the *N*-monomethyl moiety had already been reported as important in the killing action of the formamidines in cattle tick larvae, the possibility of a relationship between delayed dispersal and acaricidal effectiveness was examined and percentage mortality at 24 hr found to correlate positively with the rate of onset of dispersal.

Delayed dispersal was not characteristic of the responses to other acaricides such as lindane, allethrin, carbaryl, and coumaphos. In addition, the monoamine oxidase inhibitors, tranylcypromine, pargyline, and nialamide, did not induce delayed dispersal and showed no lethal effects.

INTRODUCTION

After the emergence of the formamidine, chlordimeform [C-8514 or *N'*-(4-chloro-o-tolyl)-*N*,*N*-dimethylformamidine], as an effective acaricide against the complex array of organophosphorus- and carbamate-resistant strains of the cattle tick, *Boophilus microplus*, in Australia (1), other formamidine-type compounds are now being developed for cattle tick control. Formamidines are active against engorged female ticks as measured by reduction in oviposition and egg viability (2); the most effective adulticides are also toxic to larvae (2). The most distinctive feature of the formamidines, however, is their ability to induce detachment of parasitic ticks from their hosts (1, 3). A paper on the relationship between detachment and the formamidine structure appears concurrently with this publication. Both investigations form part of an overall study aimed at elucidating the mode of action of formamidines in *B. microplus*.

During preliminary field trials with chlordimeform it was observed that after detachment ticks appeared hyperactive and tended to walk rapidly in what appeared to be random directions until they...
fell from the host; noticeable hyperactivity in the form of leg waving (3, 4) was also observed just before detachment. In mammals such behavioral responses would probably be correlated with interference by compounds, such as monoamine oxidase (MAO) inhibitors, with monoaminergic systems within the central nervous system (5, 6). This suggested that chlordimeform may be acting on a similar system within the cattle tick. Moreover, chlordimeform is an amidine and some compounds possessing the amidino moiety are recognized as strong MAO inhibitors (7). This knowledge led to the consideration of MAO inhibition as a possible mode of action of chlordimeform (8).

As the above theory of MAO inhibition was partly based on the hyperactive response of ticks to chlordimeform, it was decided to study this response in more detail. This paper reports the establishment of a bioassay to measure hyperactivity and the use of the bioassay to evaluate structure-activity relationships with formamidines, related nonformamidines, formamidine metabolites, conventional acaricides, and classical MAO inhibitors.

**MATERIALS AND METHODS**

**Chemicals**

The formamidines and related compounds evaluated are listed in Table 1
TABLE 2

<table>
<thead>
<tr>
<th>Chemical Structures for Chlordimeform and Related Compounds</th>
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<tbody>
<tr>
<td>I    BTS-27271</td>
</tr>
<tr>
<td>II   C-8520</td>
</tr>
<tr>
<td>III  BTS-27419</td>
</tr>
<tr>
<td>IV   BTS-23376</td>
</tr>
<tr>
<td>V    Chlordimeform or C-8714</td>
</tr>
<tr>
<td>VI   H-20013</td>
</tr>
<tr>
<td>VII  C-10405</td>
</tr>
<tr>
<td>VIII C-9140</td>
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<tr>
<td>IX   C-17296</td>
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along with their sources and designations; their chemical structures are shown in Table 2. 4'-Chloro-o-formotoluidide was provided by CIBA Co. Pty. Ltd., Australia. Tranylcyromine sulfate was obtained from Smith Kline and French Laboratories (Australia) Ltd. and pargyline hydrochloride from Abbott Australasia Pty. Ltd. The
free bases of BTS-27271, H-20013, tranylcypromine and pargyline were prepared by treating their respective salts with aqueous potassium hydroxide; these compounds were evaluated in their free base form. Piperonyl butoxide was provided by William Cooper and Nephews (Australia) Pty. Ltd. Nialamide was available as the free base.

Assay Technique

The bioassay was based on the negatively geotactic behavior of B. microplus larvae (9) and 14- to 18-day-old larvae of the acaricide-susceptible Yeerongpilly strain were used exclusively. Approximately 100 larvae were brushed onto a vertically held, sealed, capillary pipet and allowed at least 1 hr to form a stable aggregation at the tip. An application of 3 \times 2 \mu l of acetone solution of the candidate compound was made to the aggregation, the base of the pipet taped with 27-mm double-sided adhesive and the assay held at controlled room temperature and humidity (25° and 40–50% relative humidity). Larval behavior was recorded over a 24-hr period and each chemical evaluated at 2-fold serial dilutions from 0.8 to 0.003125\%. Leg waving was recorded as either a positive or negative response. Dispersal was measured as movement beyond a point 2 cm below the aggregation site. Immediate dispersal was scored 6 min after application as ++ (very active), + (slightly active), or − (inactive). Delayed dispersal was evaluated at a concentration of 0.0125\%; percentage of dispersal was plotted against log time and the resulting eye-fitted straight line used to obtain a time for 50\% dispersal (DT50). A separate sample was used for each time at which dispersal was measured and each reported DT50 is the mean of at least three replicates.

Chemical structures for this and other compounds mentioned throughout the paper are shown in Table 2.

Studies with Piperonyl Butoxide

The effect of piperonyl butoxide on DT50 values of certain compounds was examined. Aggregations were treated with 3 \times 2 \mu l of 0.003% piperonyl butoxide in acetone 60 min prior to application of the compound under examination.

Mortality Determinations

Larvicidal activity of the compounds listed in Table 1 has been reported elsewhere (2). However, it was difficult to make direct comparisons of results obtained by the different techniques of continuous exposure to impregnated packets for 48 hr and application to aggregations of larvae. Therefore, larval aggregations were treated with 0.0125% solutions as above and after a 3-min interval allowed for evaporation of the acetone, the larvae were transferred to organza-covered glass vials and held at the controlled temperature and humidity for 24 hr. Mortality was then determined, larvae that showed no movement, when observed under a low-power microscope and touched with a needle, being counted as dead. Data are means of six replicates.

RESULTS

Effect of Formamidines and Related Compounds on Activity

Immediate dispersal and inactivation were the common responses of aggregated larvae immediately after treatment; both effects were usually produced by the same compound with inactivation dominant at higher concentrations. A plot of percentage dispersal at 6 min against concentration for chlordimeform (Fig. 1) demonstrates clearly how immediate dispersal was reduced at higher concentrations presumably due to this inactivation. With higher concentrations of some compounds larvae also fell from the pipet. Immediate dispersal responses shown in Table 1 are those observed at 0.05%; at this and lower concentrations larvae neither fell nor were inactivated but
generally recovered from dispersal and re-aggregated at the tip of the pipet. With the true formamidines the effect on immediate dispersal varied depending on substitution at either the amino nitrogen or aryl moiety. With the exception of \( N'-(2,4\text{-dimethylphenyl})-N\text{-methylformamidine} \) (BTS-27271) and demethylchlordimeform (C-8520) all 4-chloro-o-tolyl alkyl and dialkyl formamidines were active. As evidenced by C-10405, C-17296, and C-17294, substitution of bromine for chlorine in the four position of the aryl group removed activity. The triazapentadienes, BTS-27419 and BTS-23376, and the thiourea, C-9140, were also inactive.

A further dispersal effect was produced by compounds I-XII (Table 1); this dispersal was delayed and usually followed recovery, i.e., reaggregation, from any immediate dispersal effect. Dispersed larvae in this instance walked randomly up and down until finally becoming immobilized in the tape at the base of the pipet. Most rapid delayed dispersal was effected by the \( N\text{-monomethyl formamidines} \), BTS-27271 and demethylchlordimeform, which produced \( DT_{50} \) values (Table 1) under 3.5 min. Other compounds which caused delayed dispersal in less than 24 hr had structures consistent with metabolism to either BTS-27271 or demethylchlordimeform or a related \( N\text{-monomethyl analog}; for those not producing this dispersal within 24 hr such metabolism is improbable. An accurate \( DT_{50} \) for the thiourea, C-9140, could not be determined. In contrast to other compounds producing delayed dispersal, C-9140 at 0.012% caused larvae to fall as well as disperse. Approximately 60% of the larvae had fallen off in 14 hr; for the remaining larvae 50% dispersal was recorded at 16.27 hr.

Leg waving was characteristic only of larvae treated with higher concentrations of either BTS-27271 or demethylchlordimeform. With both compounds leg waving developed slowly, i.e., 1–2 hr posttreatment, but then continued for some hours. This onset of prolonged leg waving coincided with the recovery of larvae from inactivation produced immediately after treatment. Little fall-off or dispersal was associated with this pronounced form of hyperactivity. All larvae displaying leg waving were dead within 48 hr, some still remaining in situ.

**Antagonism of Delayed Dispersal**

A concentration of 0.003% of piperonyl butoxide was found most suitable for this assay; at higher concentrations piperonyl butoxide itself produced either marked immediate dispersal or inactivation. Results in Table 1 show that piperonyl butoxide only antagonized delayed dispersal produced by \( N\text{-d-all-methyl formamidines and the related} N\text{-dimethyl amidine, C-17007} \). 

**Lethal Effects of Formamidines and Related Compounds**

Figures for percentage larval mortality at 24 hr are also given in Table 1. The most effective compounds were I–VI; lesser activity was displayed by compounds VII–XI; the remaining compounds gave no kill. These results confirm those of Knowles and Roulston (2) who also established compounds I–XI as the most active larvicides.

**Responses to Other Compounds**

Larval responses to 4-chloro-o-toluidine and 4′-chloro-o-formotoluidide, both metabolites of chlordimeform in *B. microplus* larvae (10), were investigated. Both were nonlethal and yielded \( DT_{50} \) values in excess of 24 hr; both metabolites produced slight immediate dispersal. Leg waving was not observed.

Neither delayed dispersal nor leg waving was observed with other types of acaricides; those studied included an organochlorine compound (lindane), a pyrethroid (allethrin), a carbamate (carbaryl), and an organophosphorus compound (coumaphos).
Fig. 1. Effect of chlordimeform concentration on dispersal of aggregated larvae of B. microplus 6 min after application.

With each chemical, larvae simply fell from the pipet; with allethrin, carbaryl and lindane, at 0.0125%, all larvae were off within 15, 25, and 30 min, respectively. With coumaphos 80% of larvae were off within 4 hr. All four acaricides effected some degree of immediate dispersal.

Larvae treated with classical monoamine oxidase inhibitors, tranylcypromine, parargyline, and nialamide, displayed neither delayed dispersal nor leg waving within 24 hr. Immediate dispersal was observed but varied considerably; tranylcypromine was very active, parargyline slightly active and nialamide inactive. The three inhibitors produced no lethal effects.

**DISCUSSION**

A bioassay was successfully established in which the hyperactive responses of cattle tick larvae to various chemical groups, including the formamidines, could be more accurately evaluated. Dispersal and leg waving, the two forms of hyperactivity produced by formamidines in field trials and detachment studies, respectively, were both measurable using the technique. Dispersal was displayed in both immediate and delayed forms.

As immediate dispersal did not occur with all compounds, it was apparently not due to the shock of dosing nor to the effect of the acetone solvent-carrier. Immediate dispersal thus seems to be a direct reaction of the larvae to the test compounds. Compounds inducing this rapid effect included not only formamidine-type compounds but also formamidine metabolites, nonformamidine acaricides, MAO inhibitors, and pipexonyl butoxide.

Delayed dispersal, however, was induced only by formamidine-type compounds and, as shown in Table 1 and Fig. 2, its rate of onset is positively correlated with mortality at 24 hr. It would appear, therefore, that delayed dispersal is actually the primary behavioral response of larvae to the lethal effect. Compounds producing no delayed dispersal within 24 hr correspondingly did not kill larvae. Furthermore the same structure–activity relationship between formamidine-type compounds was observed.
with delayed dispersal as with acaricidal effectiveness. Knowles and Roulston (2) found, firstly, that the most active larvicides were those compounds with structures compatible with metabolism to \(N\)-monomethyl formamidines and, secondly, that the lethal effect produced by \(N,N\)-dimethyl compounds was antagonized with piperonyl butoxide; comparable findings were obtained with delayed dispersal. Piperonyl butoxide is thought to act by inhibiting the mixed-function oxidase system responsible for catalyzing \(N\)-demethylation and thereby blocking metabolism to the important \(N\)-monomethyl analogs (2). Delayed dispersal by compounds not requiring \(N\)-demethylation to form \(N\)-monomethyl formamidines was not antagonized by piperonyl butoxide. For example, the \(DT_{50}\) values for BTS-27419 and BTS-23376, which hydrolyze (2) to their corresponding \(N\)-monomethyl formamidines, were virtually unchanged by piperonyl butoxide.

Classical MAO inhibitors, tranlylepromine, pargyline, and nialamide, produced neither delayed dispersal nor mortality within 24 hr; this is in contrast to the activity displayed by these compounds in causing detachment of ticks from hosts (4). These results are particularly interesting in view of very recent evidence that formamidines inhibit, in vitro, both rat liver (11, 12) and cattle tick (13) MAO. This would suggest that MAO inhibition may be involved in the detaching action, but not the killing action, of the formamidines on the cattle tick. To clarify the latter it will be necessary to examine MAO levels.
of ticks treated with formamidines; if MAO inhibition is involved in killing, then we should observe a critical level of MAO, with each formamidine, at a time corresponding to the onset of lethal effects, a time to which DT₅₀ values are readily applicable.

The delayed response of prolonged leg waving produced by only high concentrations of BTS-27271 and demethylchlorodimeform is interesting in that a similar, but immediate, prolonged leg waving response on treatment with high concentrations of BTS-27271 and demethylchlorodimeform has also been observed with female ticks attached to mice (4). With larvae the onset of prolonged leg waving coincided with recovery from inactivation which suggests that in the absence of inactivation larvae may also have displayed an immediate and not a delayed response. However, it appears probable that this prolonged leg waving displayed by both larvae and female ticks is not the same leg waving response displayed by female ticks just prior to detachment (3, 4). At the concentrations of BTS-27271 and demethylchlorodimeform which produced leg waving in female ticks, detachment was inhibited (4); at those concentrations producing the same effect in larvae, dispersal was inhibited. This suggests that at higher concentrations the mode of action of the N-monomethyl formamidines is different from and interferes with that at lower concentrations.

REFERENCES


2. C. O. Knowles and W. J. Roulston, Toxicity of formamidine acaricides and related compounds to Boophilus microplus (Canestrini), and modification of toxicity by certain insecticide synergists, J. Econ. Entomol. 6, 1245 (1973).


