Novel protease inhibitors for control of sheep blowfly and other insects

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Summary
A range of aminopeptidase inhibitors were screened for activity against L. cuprina in in vitro and in vivo assays. Two classes of inhibitors were identified with significant effects on these insects in culture and in sheep trials. Several specific molecules were identified and are now undergoing field trials. Combinations of these molecules with other protease inhibitors or other insecticidal molecules suggests significant potential for new control protocols and products.

Keywords
Lucilia cuprina, sheep blowfly, aminopeptidases, proteases, inhibitors, bioassays, myiasis.

Introduction

Work in this laboratory in the 1990's established that aminopeptidases were potential targets for the control of a range of insects including the sheep blowfly, *Lucilia cuprina* (Reed *et al*., 1999). Studies suggested that the blowfly was susceptible to various aminopeptidase inhibitors but that those targeting leucine aminopeptidase were particularly effective. In addition the combination of these inhibitors with inhibitors of trypsin-like serine proteases could result in synergistic effects to virtually stop larval growth in *in vitro* culture. These findings were patented and funding successfully sought under the START initiative to continue the work of defining compounds that were inhibitors of insect aminopeptidases and of insect growth. This paper outlines the resultant work which enabled the identification of aminopeptidase inhibitors that are effective against the sheep blowfly and other insects.

Methods

Enzyme inhibitors
Enzyme inhibitors were produced under contract with CSIRO Molecular Sciences at Clayton Victoria (Table 1). A range of compounds were synthesised in small amounts and tested for both AP inhibitory activity and ability to inhibit larval growth. These compounds were designed to be similar in structure to a variety of known mammalian aminopeptidase inhibitors. Analogues and synthesis intermediates of many of the compounds were also screened. Included in the screening protocol were derivatives of N-arylphthalimides and N-arylhomophthalimides, which are known to inhibit aminopeptidase N (Miyachi *et al*., 1998), and aminotetralones, which are inhibitors of aminopeptidase M (Schalk *et al*., 1994). Synthesis precursors of the aminopeptidase M inhibitor actinonin (Umezawa *et al*., 1985) were also screened. A range of amino acid-based hydroxamic acid derivatives were examined, based on previous studies showing that related compounds were able to inhibit aminopeptidases (Chan *et al*., 1982; Baker *et al*., 1983; Wilkes and Prescott, 1983). Methylphosphonic acids were tested based on data showing that phosphonic acid derivatives of amino acids were effective inhibitors of porcine kidney leucine aminopeptidase (Giannousis and Bartlett, 1987) and that the addition of a methylene group to this type of compound can render it more resistant to hydrolysis (Spengler and Burger, 1998). The initial screening protocol showed that hydroxamic acid and methylphosphonic acids were the most effective compounds, and compounds incorporating both these groups were synthesised and tested. Potential inhibitors were supplied in crystallised form and were dissolved in a suitable solvent, usually a buffered saline before testing in an enzyme assay and in *in vitro* growth assays against *Lucilia cuprina*.

Insects
Insect colonies were housed independently in constant temperature rooms (25-27˚C) with a 16/8 hour light/dark cycle. *L. cuprina* eggs were obtained from a laboratory colony after oviposition on liver.

Eggs were sterilised and hatched by standard techniques before growing the larvae on a defined medium (Reed *et al*., 1999). Care was taken at all stages of the rearing process to limit the potential for microbial contamination.

Enzyme preparations

Enzyme preparations were obtained from third instar larvae of *Lucilia cuprina*. Sheep blowfly aminopeptidase activity was found to be at high levels in Larval Excretory Secretory Products (LESP), the preparation of which has been previously described (Sandeman *et al*., 1990).

Aminopeptidase inhibition assay

Aminopeptidase inhibitory potential was assessed using leucine-p-nitroanilide and the leucine aminopeptidase enzyme source sheep blowfly LESP. Endpoint assays were first performed to determine the appropriate dilution of enzyme to use. Inhibition assays were performed in 96 well microtitre trays blocked with 0.1 M glycine and washed in PBS/Tween prior to assay. Each well contained 50 µl assay buffer (0.1 M Tris-HCl, 5 mM $MgCl_2$, pH 8.0), 50 µl enzyme preparation and 50 µl inhibitor, and plates were incubated at 25˚C for 30 minutes. Substrate (50 µl) was added to a final concentration of 1.5 mM, and the plate was incubated for a further 15 minutes at 25˚C before monitoring liberation of p-nitroaniline by reading absorbance at 405 nm. Inhibitors were each tested in duplicate at final concentrations of 1.25, 2.5, 5 and 10 mM. Controls without substrate and without enzyme were included in each assay to correct for any colour present due to the enzyme or the inhibitor, respectively. A positive inhibitory control (the commercial aminopeptidase inhibitor, actinonin) was also included on each plate, and results for each inhibitor were expressed as a ratio relative to this positive inhibitory control. Where the ratio assumed a negative number it was adjusted to zero, so the least effective inhibitors have a ratio of zero and the best inhibitors have a ratio greater than one.

Media bioassay

This assay was performed to investigate the efficacy of potential aminopeptidase inhibitors in inhibiting larval growth. Media was prepared as described above and freshly hatched *Lucilia* larvae were grown on 1 ml of media with 100 µl of test compound. Fifty larvae were placed in each McCartney bottle with at least 5 replicates for each treatment and controls. Each compound was tested at 1.25, 2.5, 5 and 10 mM final concentration. After 24 hrs the larvae were frozen at –20°C before removal and weighing.

Sheep Trials

Two sheep experiments were undertaken to test the efficacy of selected aminopeptidase inhibitors in treating flystrike on sheep. Each trial consisted of 34 merino wethers kept in individual pens in the sheep house at La Trobe University. The sheep were fed a mixture of sheep pellets, lucerne and oaten chaff to 1.5 times their daily requirements. Sheep were each infected on the backs with 6 individual groups of 300 larvae by first wetting each site with 200 mls of distilled water then placing the larvae on two soaked dental plugs and applying these to the skin between the wool staple. The staple was then held around the plugs by a rubber band. Test chemicals were applied to the dental plugs just before application of the larvae and in a planned randomized pattern across all sites on all sheep. At least 6 replicates were made of each treatment. The infections were allowed to develop for 48 hours before they were removed by shearing the site. The number and size of the larvae were scored (1= freshly hatched larvae; 5= 3rd instar greater than 10 mm long) and the skin wound was measured. Sheep were treated with a proprietary blowfly dressing and allowed to recover.

Results

Enzyme Assays

Aminopeptidase assays carried out with the various manufactured compounds showed that the arylhomothalimides, tetralones and arylthalimides were poor insect aminopeptidase inhibitors when compared to the commercial inhibitor, actinonin. In comparison the methylphosphonates, hydroxamates and actinonin precursors did show a number of compounds with inhibitory activity at least equal to actinonin (Figure 1). The most promising groups were the methylphosphonic acids

either alone or in combination with hydoxamic acids and several of these were selected for bioassay studies.

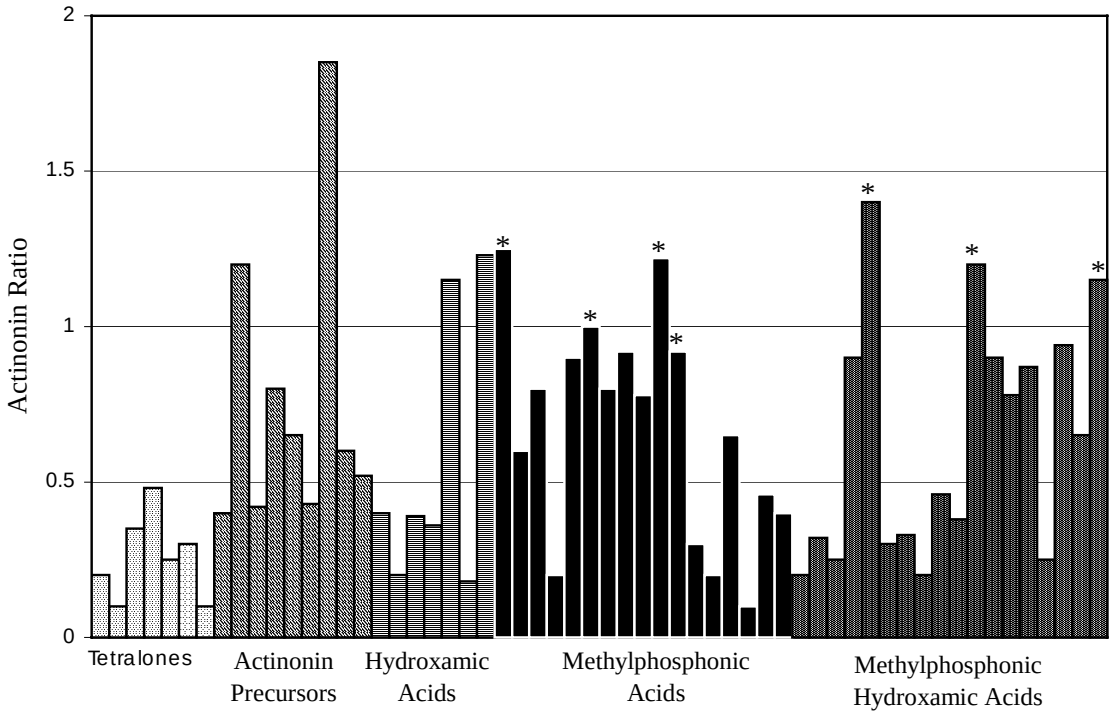

Figure 1. Blowfly aminopeptidase inhibition by various derivatised amino acids (10mM) by comparison with actinonin. An actinonin ratio >1 is a better inhibitor than actinonin at the same molarity. * selected for further testing.

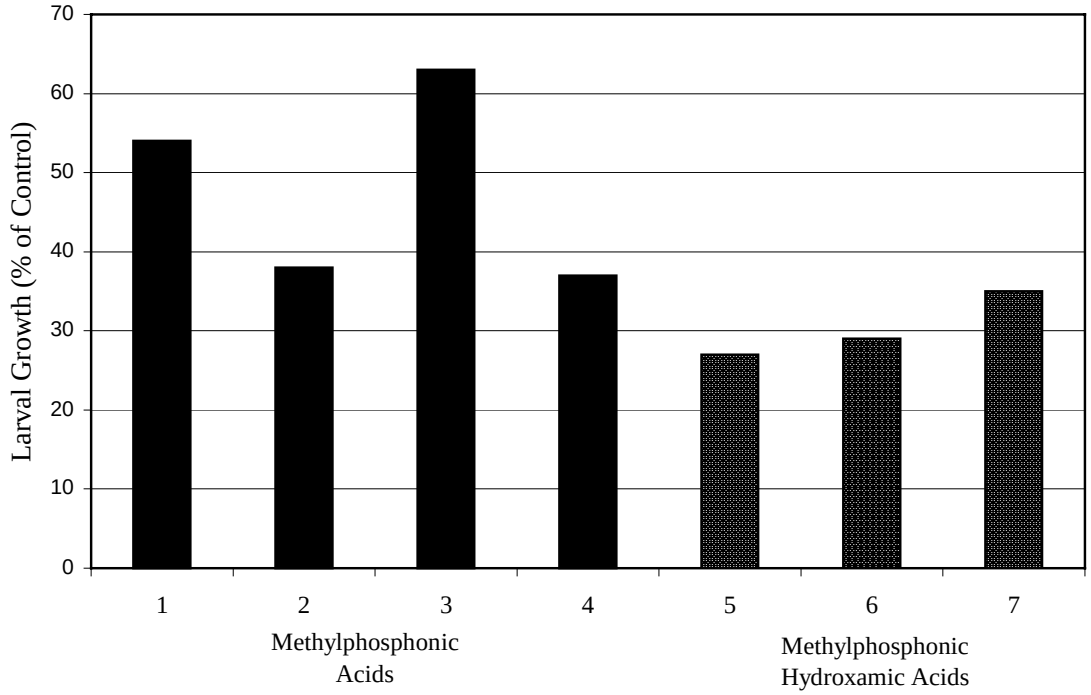

Figure 2. Growth of Lucilia cuprina larvae on a defined media in the presence of various compounds (10mM) as a percentage of the media control.

Media Bioassays

Bioassays analysing larval growth in defined media confirmed the activity of the methyl phosphonates in inhibiting larval development in both species (Figure 2). However, it was clear that high levels of aminopeptidase inhibition in the enzyme assay did not necessarily predict inhibitory activity against the whole larvae.

Although the methylphosphonic hydroxamic acids appeared marginally more inhibitory than the methylphosphonates the difference was not significant and the more complicated manufacturing protocol led to selection of the No. 4 methylphosphonic acid (MPA4) (Figure 2) as our lead molecule.

MPA4 was then compared to EDTA, a general inhibitor of metalloenzymes and TLCK a specific trypsin inhibitor in isolation or in combination in *in vitro* larval growth assays. The level of inhibitor was varied to determine the existence of any additive or synergistic effects. The results suggest that although inhibition with the single compound at a particular concentration may not be significant, combinations have a much greater effect and that TLCK, which is a poor inhibitor of growth *in vitro*, is a particularly interesting addition to a combined treatment (Figure 3).

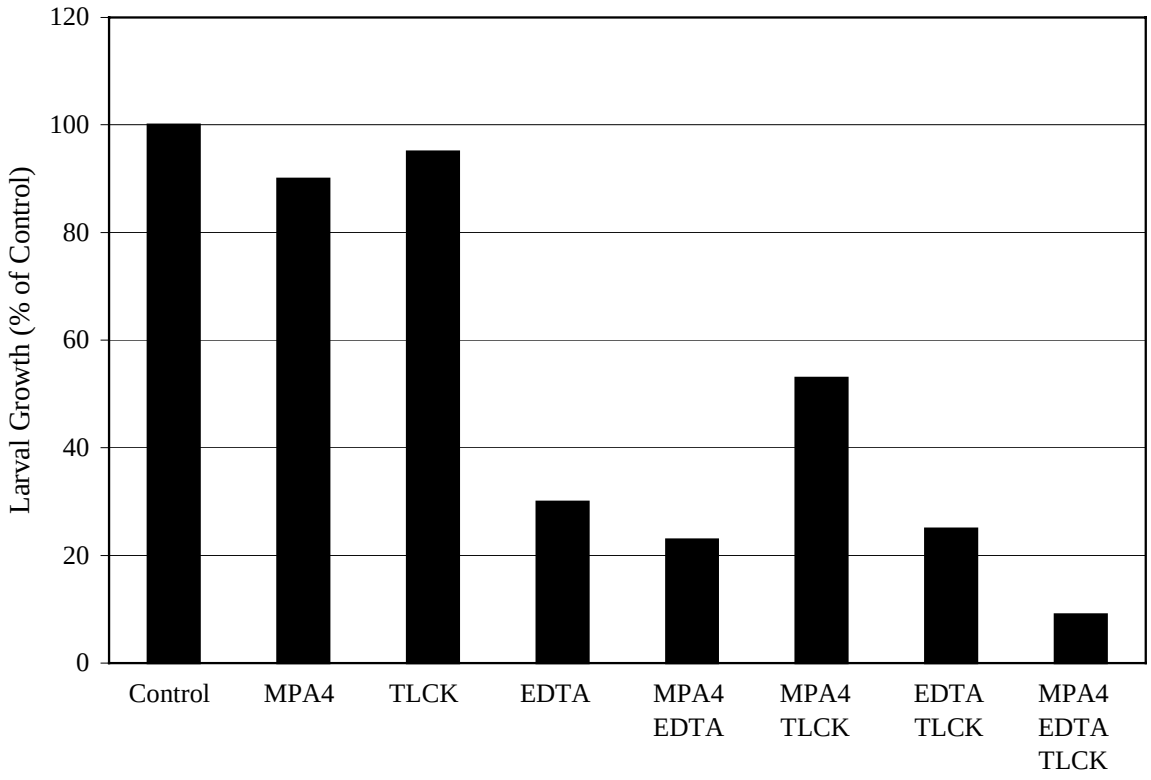

Figure 3. Growth of Lucilia cuprina larvae on a defined media in the presence of enzyme inhibitors and mixtures of inhibitors (2.5mM each) as a percentage of the media control.

Sheep Assays

Two sheep trials were carried out with various inhibitors in isolation or in combination. The results of the second trial are presented here. Six replicates were carried out testing each compound or combination and 24 replicates were buffer controls. Results are presented in Figures 4, 5 and 6.

The number of sites which were successfully infected are shown in Figure 4. It is clear that usually infection rates of around 80% can be achieved with the implant technique. The only groups to show lower infection rates were the MPA4/EDTA and the MPA4/EDTA/TLCK combinations.

In contrast, maggot size though variable, was lower than the control in the single treatments, EDTA and MPA4 and in the combinations EDTA/MPA4 and EDTA/TLCK (Figure 5). The combination of

the three inhibitors had a low mean larvae size but a large amount of variation between sites. TLCK had little effect on maggot size.

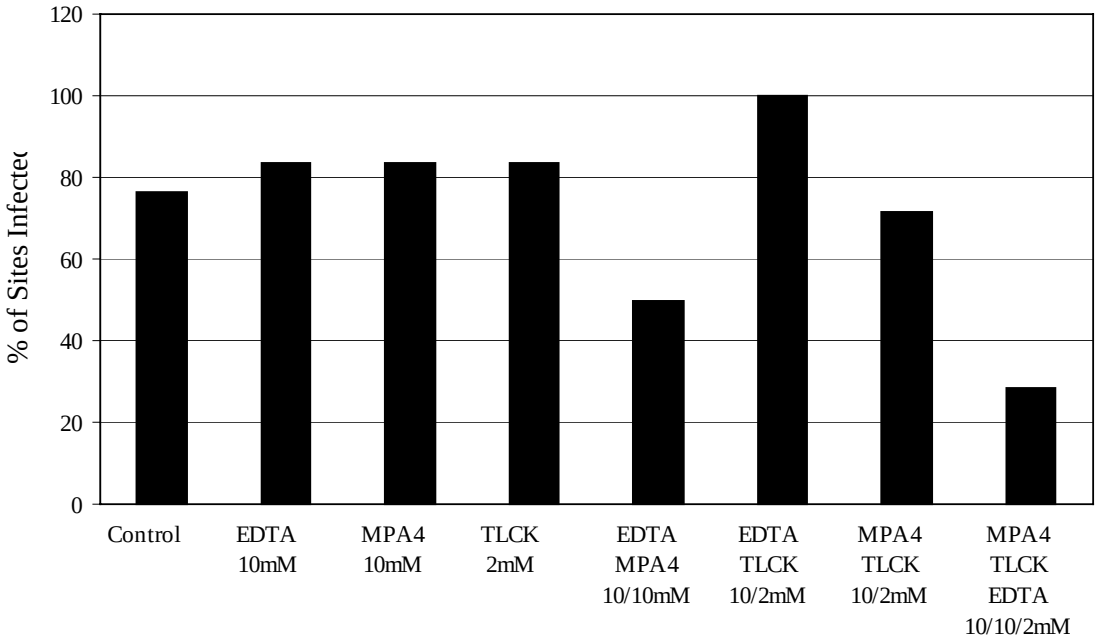

Figure 4. Sites infected (% of total sites) on sheep implanted with 300 larvae per site in the presence of various enzyme inhibitors or the buffer control.

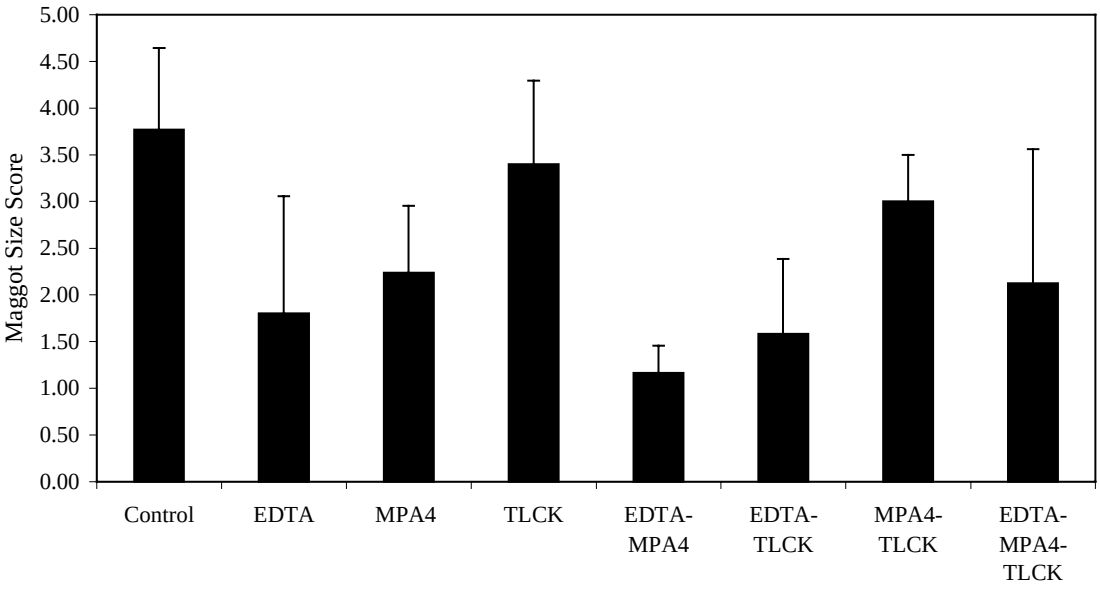

Figure 5. Size of maggots from sites infected with 300 larvae in the presence of various enzyme inhibitors or the buffer control.

Wound size was again very variable between sites (Fig. 6) with the smallest sites receiving MPA4, EDTA/MPA4, EDTA/TLCK and the triple combination a low level of variability. TLCK was again ineffective.

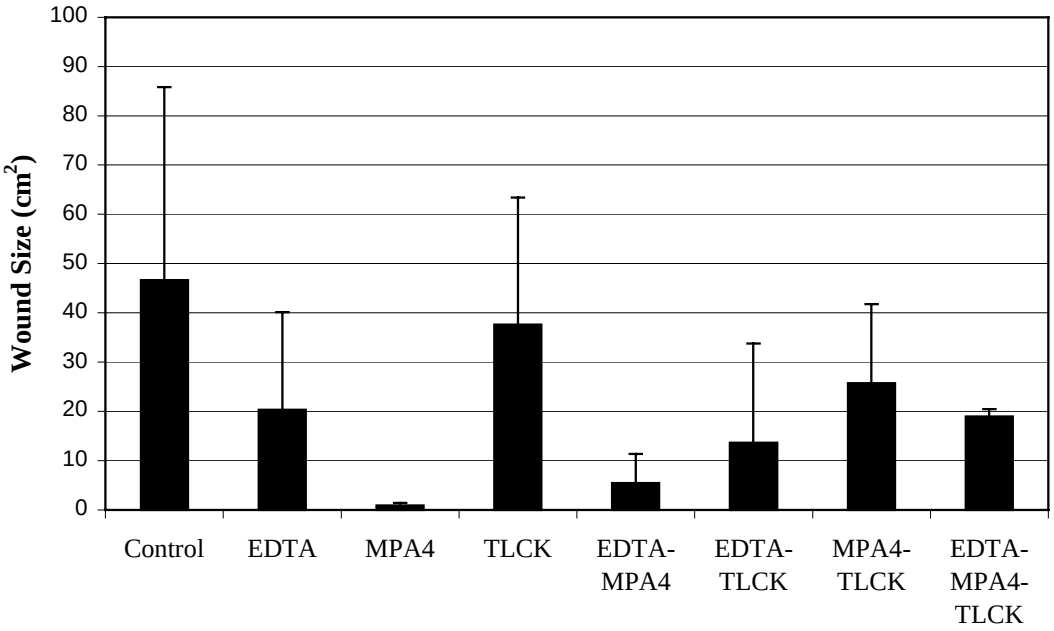

Figure 6. Wound size of infections 48 hours after larval implant with 300 larvae and various enzyme inhibitors.

Discussion

This paper reports the successful identification and testing of an aminopeptidase inhibitor that is also an effective inhibitor of *L. cuprina* larval growth *in vitro* and reduces larval survival and the impact of infection *in vivo*. Studies of a range of potential aminopeptidase inhibitors suggested that the methylphosphonic acids and hydroxamic derivatives of various amino acids were at least as potent as the commercial, bacterial product, actinonin. These compounds have the advantage of being relatively easily manufactured and are not residual, breaking down to biologically inert molecules. One of the methylphosphonic acids was selected for further studies based on its effectiveness against aminopeptidases and its ability to affect the growth of larvae of *Lucilia cuprina* and the cotton bollworm, *Helicoverpa armigera*.

MPA4 was tested alone or in combination with other metallopeptidase and serine protease inhibitors. These studies suggested that MPA4 was most effective at concentrations around 10mM and that combinations with EDTA and/or the trypsin inhibitor, TLCK, could produce synergistic effects at certain concentrations. Interestingly, TLCK alone was not an effective inhibitor of larval growth although it does inhibit gut trypsin activity. When tested in bioassays in combination with MPA4, or with MPA4 and EDTA, TLCK significantly added to the inhibition of larval growth. This effect would suggest that trypsin is not of major importance in gut digestion in *L. cuprina* unless there is concomitant inhibition of aminopeptidases. Thus trypsins may be able to replace aminopeptidase activity at the stage of terminal digestion of protein in the gut of this dipteran. The additive effect of EDTA also suggests that aminopeptidases are not the only class of metallopeptidases active in gut digestion though other studies in our laboratory indicate that they are responsible for the majority of amino acid production.

The additional effects of combinations of these inhibitors was confirmed by the experiments on the establishment of *L. cuprina* infections on sheep. The best inhibitor combination over all the indices of infection success, wound size, maggot size and percentage infected, was MPA4/EDTA. In the presence of this combination 3 of 6 infections established, maggot size was close to 1 (the size of a freshly hatched first instar) and the wound size was 5 cm^2. The next best combination was probably MPA4/EDTA/TLCK and clearly the addition of TLCK did not assist in reducing maggot or wound size. However, TLCK did seem to have some effect on infection establishment since the triple combination was associated with the lowest level of infection, 1 of 6 sites infected. This dichotomy

in the effects of TLCK might be explained by a role for trypsin-like proteases in wound establishment in the first instar but not in larval nutrition or wound expansion. Wound formation is a critical phase in larval establishment especially using this method of larval implantation (Sandeman *et al*., 1987). The lack of effect of TLCK on wound and maggot size *in vivo* suggests that the *in vitro* finding of an effect on larval growth may not occur *in vivo* perhaps because of the differing nutrient sources.

The other interesting finding is the effect of EDTA and especially MPA4 alone on wound size and to a lesser extent maggot size. Clearly, both these inhibitors affect larval growth *in vitro* and *in vivo* and this effect would seem to be directly correlated to wound size *in vivo*. Thus, inhibiting larval growth also slows wound formation, a combined effect that might significantly add to the protective ability of any treatment arising from the use of such inhibitors.

Additional work not reported here has confirmed the effects of MPA4 on other insect larvae notably the cotton bollworm. As a result a program of field trials have now begun to check the potential of MPA4 in insecticidal applications.

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