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Insecticidal activities of histone deacetylase inhibitors against a dipteran parasite of sheep, *Lucilia cuprina*

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ABSTRACT

Histone deacetylase inhibitors (HDACi) are being investigated for the control of various human parasites. Here we investigate their potential as insecticides for the control of a major ecto-parasite of sheep, the Australian sheep blowfly, *Lucilia cuprina*. We assessed the ability of HDACi from various chemical classes to inhibit the development of blowfly larvae *in vitro*, and to inhibit HDAC activity in nuclear protein extracts prepared from blowfly eggs. The HDACi prodrug romidepsin, a cyclic depsipeptide that forms a thiolate, was the most potent inhibitor of larval growth, with equivalent or greater potency than three commercial blowfly insecticides. Other HDACi with potent activity were hydroxamic acids (trichostatin, CUDC-907, AR-42), a thioester (KD5170), a disulphide (Psammaplin A), and a cyclic tetrapeptide bearing a ketone (apicidin). On the other hand, no insecticidal activity was observed for certain other hydroxamic acids, fatty acids, and the sesquiterpene lactone parthenolide. The structural diversity of the 31 hydroxamic acids examined here revealed some structural requirements for insecticidal activity; for example, among compounds with flexible linear zinc-binding extensions, greater potency was observed in the presence of branched capping groups that likely make multiple interactions with the blowfly HDAC enzymes. The insecticidal activity correlated with inhibition of HDAC activity in blowfly nuclear protein extracts, indicating that the toxicity was most likely due to inhibition of HDAC enzymes in the blowfly larvae. The inhibitor potencies against blowfly larvae are different from inhibition of human HDACs, suggesting some selectivity for human over blowfly HDACs, and a potential for developing compounds with the inverse selectivity. In summary, these novel findings support blowfly HDAC enzymes as new targets for blowfly control, and point to development of HDAC inhibitors as a promising new class of insecticides.

1. Introduction

The Australian sheep blowfly (*Lucilia cuprina)* is an important ecto-parasite that causes fly strike, which has significant health and welfare, as well as economic, impacts on the sheep industry in Australia (Sandeman et al., 2014). The female blowfly is attracted to the sheep by odours, particularly those associated with bacterial infections in damp fleece, and lays eggs (Tellam and Bowles, 1997). The developing larvae feed on the sheep, causing severe tissue damage, toxaemia, and in some cases, death. The consequent loss of livestock, costs of preventative and curative chemical treatments, and animal welfare issues place significant economic burdens on livestock enterprises (Lane et al., 2015). The blowfly has developed resistance to various classes of chemical insecticides used for its control, including organochlorines, organophosphates, the benzoyl-phenyl urea diflubenzuron (Levot, 1995; Sandeman et al., 2014) as well as the triazine cyromazine (Levot, 2012). Only two preventative blowfly control chemicals, the macrocyclic lactone ivermectin and the cyanopyrimidine dicyclanil, remain effective with no resistance yet reported. There is therefore a need to identify new chemical classes of insecticides, preferably with different target proteins, to control this important parasitic insect.

Histone deacetylase inhibitors (HDACi) have been recognised as therapeutic targets in cancer for many years (Cairns, 2001), with a

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number in clinical use or clinical trials as anti-cancer drugs. They have also been studied extensively over recent years for their potential in chemotherapy for parasitic diseases of humans, including malaria, toxoplasmosis, trypanosomiasis, schistosomiasis and leishamaniasis (Andrews et al., 2012a,b; Marek et al., 2015). HDAC enzymes have been studied extensively in the model dipteran insect *Drosophila* with respect to their roles in longevity and memory formation (Fitzsimons et al., 2013; Proshkina et al., 2015; Schwartz et al., 2016), with a *Drosophila* model providing experimental evidence to highlight HDACi as potential therapeutics for the treatment of Huntington's disease (Sharma and Taliyan, 2015). However, only a single study has reported the insecticidal activity of an HDACi against this fly species, with Pile et al. (2001) noting that trichostatin caused lethality during larval development. The potential for HDACi as insecticides was recently highlighted by Kotze et al. (2015) who showed that trichostatin and suberoylanilide hydroxamic acid (SAHA) were able to inhibit the development of sheep blowfly larvae *in vitro*. That report also highlighted similarities and differences in amino acid sequences of blowfly and human HDAC enzymes, with differences particularly noted between species for the Class II enzymes HDAC4 and 6, and the Class IV HDAC11, raising the possibility of identifying insect-specific inhibitors.

The present study expands on our earlier report of insecticidal activity for trichostatin and SAHA (Kotze et al., 2015) by examining other HDACi with different chemical structures and mechanisms of action. We focus on hydroxamic acids since these are the best known group of HDACi, but also include inhibitors with different chemical components, such as benzamides, thioesters, thiolates, disufides, cyclic depsi- and tetra-peptides, fatty acids, and sesquiterpene lactones (Table 1). We measure the effects of these HDACi on the development of blowfly larvae (larval growth rate and pupation rate) and on the HDAC enzyme activity of nuclear protein extracts prepared from blowfly eggs. We also compare these results with reported inhibitory activities against human HDAC enzymes as an initial step towards identification of insect-specific inhibitors.

2. Materials and methods

2.1. Insects and chemicals

The *L. cuprina* used in this study were from the laboratory reference drug-susceptible LS strain, derived from collections made in the Australian Capital Territory (Canberra, Australia) over 40 years ago. This strain has been maintained in a laboratory since that time (in Canberra for 30 years, and then at CSIRO and University of Queensland laboratories in Brisbane for the last 10 years), and has no history of exposure to insecticides. Adult flies were maintained at 28 °C and 80% relative humidity with a daily photoperiod of light 16 h and dark 8 h. Adults were fed a diet of sugar and water, while larvae were raised on a wheatgerm culture medium (Tachibana and Numata, 2001). Protein meals (bovine liver) were provided on days 4 and 8 after adult eclosion in order to prime adult flies for subsequent egg-laying. For provision of eggs for bioassays, liver was placed into cages of gravid flies for a period of two hours (12 p.m. until 2 p.m.). The liver was then removed and kept at room temperature overnight. At 10 a.m. the next morning, assays were established using the newly-hatched larvae.

HDACi were synthesized by reported procedures or obtained from commercial sources (Table 1). The structures are shown in Supplementary Figs. 1–4. Stock solutions for use in larval bioassays were prepared in ethanol at a concentration of 1 mg/mL. In cases where the compound did not dissolve at this concentration the solutions were further diluted 2-fold with ethanol until no precipitate was evident (to give stocks at 0.5 or 0.25 mg/mL). Exceptions were CUDC-907 and MC1568 which required dilution to a concentration of 0.05 mg/mL. The commercial insecticide stocks used as controls were prepared at 1 mg/mL in water (cyromazine and dicyclanil) or acetone (diflubenzuron). Stock solutions of HDACi for use in nuclear extract HDAC enzyme assays were prepared at 1 mg/mL in DMSO.

2.2. Blowfly larval bioassay

The effects of HDACi on the growth of blowfly larvae was assessed using a bioassay system in which larvae were allowed to develop on cotton wool impregnated with the compounds at various concentrations (modified slightly from Kotze et al., 2014). Briefly, 4 mL aliquots of HDACi or commercial insecticide solutions were added to cotton wool plugs and the solvent (4 mL of either ethanol, acetone, or water) was allowed to evaporate overnight. Control containers were prepared by addition of 4 mL of the relevant solvent to the cotton wool. The next day (Day 0 of the assay), a sheep serum-based medium (80 g/L yeast extract (Merck), 1.6 mg/mL tylosin (Sigma) in lamb serum (Life Technologies) buffered with 35 mM KH_2PO_4, pH7.5) was added to the cotton wool, and groups of 50 freshly-hatched larvae (prepared as described in section 2.1, above) were placed onto the cotton wool. The assay pots were placed at 28 °C. In order to calculate mean larval weight at the beginning of the drug exposure period, two groups of 100 larvae were collected, blotted dry on paper towel, weighed and discarded on Day 0. After 24 h (Day 1), 3 larvae were removed from each container, weighed, and discarded. The remaining larvae were fed with 1 mL of nutrient medium on Day 1, and then 2 mL on each of Days 2 and 3. Late on Day 4, the containers were placed into larger pots with a layer of sand at the base to serve as a medium for pupation, and returned to the incubator. Pupae were recovered from the sand on sieves on Day 9, and counted.

Each compound was examined at four or five serially diluted (5-fold) concentrations. Each experiment consisted of a single container at each concentration of HDAC inhibitor or insecticide, alongside 4 control assays. Two separate experiments were performed for each compound. The effect of the compounds on larval development was defined in two ways:

i) Larval weight gain in first 24 h; the total weight gain of the 3 larvae sampled on Day 1 was expressed as a percentage of the mean of the weight gain of the 3 larvae sampled from each of the 4 control containers (weight gain was calculated by difference using weight on Day 1 and the mean weight of larvae on Day 0);
ii) Pupation rate; the number of pupae in each drug-treated container was expressed as a percentage of the mean number of pupae in the 4 control containers.

The larval weight and pupation rate dose-response data were analysed with GraphPad Prism® software using non-linear regression, with the ‘variable slope’ option selected, in order to calculate IC_{50} values (with 95% Confidence Intervals) representing the concentration of inhibitor required to reduce the larval weight gain or pupation rate to 50% of that measured in control (no drug) treatments.

2.3. Nuclear extract preparation

Nuclear extracts were prepared from blowfly eggs (0.5 g) using a Nuclear Extraction kit (Millipore, USA) following the manufacturer's protocol with some modifications. The chorion was removed by soaking for 80 s in a solution of bleach (2% v/v), followed by centrifugation to sediment the eggs. The eggs were washed 3 times in ice cold PBS. Complete Mini Protease Inhibitor (Roche, Basel

Table 1
HDAC inhibitors and insecticides used.

Drug group	Compound	Human HDACs Inhibited	References	Source
1) HDAC inhibitors				
Hydroxamic acids	Trichostatin	Class I and II	Yoshida et al., 1995	Selleckchem
	CUDC-907	Class I and II; also class I PI3K	Qian et al., 2012	Selleckchem
	AL1179-3b	Class I and II	Kahnberg et al., 2006	synthesized
	AR-42	Class I and II	Lu et al., 2005; Tseng et al., 2015	ApexBio
	Quisinostat	Class I and II	Arts et al., 2009	Selleckchem
	PG50	HDAC6	Gupta et al., 2010	synthesized
	Nexturastat A	HDAC6	Bergman et al., 2012	ApexBio
	AL1179-84	Class I and II	Kahnberg et al., 2006	synthesized
	Panobinostat	Class I and II	Atadja, 2009; Rajkumar and Kumar, 2016	ApexBio
	Pracinostat (SB939)	Class I and II	Novotny-Diermayr et al., 2010	ApexBio
	SBHA	Class I and II	Richon et al., 1998	ApexBio
	AL-1179-85	Class I and II	Kahnberg et al., 2006	synthesized
	SAHA (Vorinostat)	Class I and II	Richon et al., 1998; Iwamoto et al., 2013	ApexBio
	Givinostat	Class I and II	Leoni et al., 2005	ApexBio
	M344	Class I and II	Heltweg et al., 2004	ApexBio
	Resminostat	Class I and II	Mandl-Weber et al., 2010	ApexBio
	Belinostat	Class I and II	Plumb et al., 2003; Thompson, 2014	ApexBio
	Naphthohydroxamic acid	HDAC8	Krennhrubec et al., 2007	Sigma-Aldrich
	Droxinostat	Class I and II	Wood et al., 2010	ApexBio
	CAY10603	Class I and II	Kozikowski et al., 2008	Santa Cruz Biotech
	VAHA (Valproic acid hydroxamate)	Class I and II	Fass et al., 2010	Santa Cruz Biotech
	MC-1568	Class IIa	Mai et al., 2005	Selleckchem
	ABHA	Class I and II	Andrews et al., 2000	synthesized
	NW58	HDAC 1& 2	Wheatley et al., 2010	synthesized
	Tubacin	HDAC6	Butler et al., 2010	Selleckchem
	HPOB	HDAC6	Lee et al., 2013	ApexBio
	BRD73954	HDAC6 and HDAC8	Olson et al., 2013	ApexBio
	CUDC-101	Class I and II EGFR (epidermal growth factor receptor) HER2 (human epidermal growth factor receptor 2)	Lai et al., 2010	ApexBio
	Rocilinostat	HDAC6	Santo et al., 2012	Selleckchem
	Tubastatin A	HDAC6	Butler et al., 2010	ApexBio
	PCI-34051	HDAC8	Balasubramanian et al., 2008	Santa Cruz Biotech
Cyclic depsipeptide	Romidepsin	Class I	Furumai et al., 2002; Barbarotta and Hurley 2015	ApexBio
Benzamides	Entinostat	Class I	Hu et al., 2003	ApexBio
	Mocetinostat	Class I	Fournel et al., 2008	ApexBio
Thioester	KD5170	Class I and II	Hassig et al., 2008	ApexBio
Disulfide	Psammaplin A	Class I	Baud et al., 2012; Kim et al., 2007	Santa Cruz Biotech
Thiolate	TCS HDAC620b	HDAC6	Suzuki et al., 2006	ApexBio
Cyclic tetrapeptide	Apicidin	HDAC1; Anti-protozoan activity	Jones et al., 2006; Darkin-Rattray et al., 1996	ApexBio
Fatty acids	Valproic acid	Class I and II	Phiel et al., 2001; Fass et al., 2010	Sigma-Aldrich
	Pivanex (AN-9)	Histone hyperacetylation	Rabizadeh et al., 2007	Sigma-Aldrich
Sesquiterpene lactone	parthenolide	Depletes HDAC1 but not other class I/II HDACs	Gopal et al., 2007	Santa Cruz Biotech
2) Commercial blowfly insecticides				
Pyrimidine	Dicyclanil	Insect growth regulator: mechanism unknown	–	Fluka
Diamino-triazine	Cyromazine	Insect growth regulator: mechanism unknown, affects cuticle extensibility	Kotze and Reynolds, 1990	Chem Service
Benzoyl phenyl urea	Diflubenzuron	Insect growth regulator: inhibits chitin synthesis	Hajjar and Casida, 1978	Chem Service

Switzerland) in PBS was added to the washed eggs before disrupting them by hand with a plastic pestle. The disrupted eggs were centrifuged at 250*g* for 1 min at 4 °C, and supernatant removed. The egg cell pellet was washed with 1000 μL of ice cold PBS, resuspended by inversion, centrifuged at 1000*g* for 5 min at 4 °C, and the supernatant removed. This wash step was repeated a further 2 times. The cells were then disrupted by drawing 5 times through a 21 g needle fitted to a 1 mL syringe. The suspension was centrifuged at 8000*g* for 20 min at 4 °C, the supernatant removed and discarded, and the pellet retained (nuclear portion). The nuclear pellet was resuspended in 2/3 of the original cell pellet volume of ice cold nuclear extraction buffer (containing 0.5 mM DTT and protease inhibitor cocktail, Millipore, Temecula). The solution was placed on low speed roller for 1 h at 4 °C, then centrifuged at 16000*g* for 5 min at 4 °C, and the supernatant (the nuclear extract) transferred to a new tube. The protein concentration was measured by the method of Bradford (1976) using the Bio-Rad protein assay reagent, and bovine serum albumin as a standard. The extract was then aliquoted into separate tubes, snap-frozen in liquid nitrogen, and stored at −80 °C.

2.4. HDAC enzyme assay

A fluorometric assay kit (Sigma-Aldrich, USA) was used to measure HDAC enzyme activity in blowfly nuclear extracts, as described in the kit instructions, except that the volumes of all reagents were reduced to give a total assay volume of 27.5 μL. Each assay contained approximately 15 μg of nuclear extract protein. HDAC activity was measured in the presence or absence of HDACi. Control assays were also run in the presence of 1.25 μM trichostatin in order to calculate the amount of fluorescent product that was derived from a trichostatin-inhibitable reaction, that is, the amount of product derived from the action of HDAC enzymes alone. The assay was performed using a series of at least 4 serially-diluted working solutions of each HDACi. Duplicate assays were performed at each HDACi concentration. The fold dilutions used to generate each working solution series varied from 2–fold to 10-fold, and were set (based on initial dose-finding experiments) in order to provide a dose response curve consisting of 4–6 data points. The % inhibition of HDAC activity was calculated for each concentration of HDACi added to the reaction. The enzyme assay dose-response data were analysed with GraphPad Prism® software using non-linear regression, with the 'variable slope' option selected, in order to calculate IC_{50} values (with 95% Confidence Intervals) representing the concentration of inhibitor required to reduce the HDAC activity of the nuclear extract by 50%.

2.5. Larval and enzyme assay comparisons

We performed a non-parametric (Spearman) correlation analysis in GraphPad Prism® in order to examine the relationship between the effects of HDACi in inhibiting blowfly larval development and inhibiting nuclear extract HDAC enzyme activity. In addition, in order to examine the relationship between the blowfly bioassay data and the reported inhibitory effects of the HDACi against specific human HDAC enzymes, we performed a correlation analysis using the bioassay data and IC_{50} values reported in the scientific literature for the HDACi against human HDAC enzymes (see Supplementary Table 1). While blowflies are known to possess HDAC1, 3, 4, 6 and 11, (Kotze et al., 2015), the analysis was only performed with human HDAC1, 3, 4 and 6 as insufficient inhibition data was available for an analysis of inhibitory effects on human HDAC11. For the correlation analysis, we grouped HDAC 1 and 3 together as Class I HDAC enzymes, and HDAC4 and 6 together as Class II HDAC enzymes.

3. Results

Forty HDACi compounds were investigated for inhibition of the growth of blowfly larvae, with their activities reported in Table 2 as inhibition of larval weight gain and pupation (μg/assay). For comparison, the toxicities of three commercial blowfly insecticides are also reported in Table 2. The most potent inhibitor of blowfly larval growth was the depsipeptide romidepsin, which was more potent, or as potent as, the commercial insecticides: 10-fold more potent than cyromazine, 2-fold more potent than diflubenzuron, and equipotent with dicyclanil (Table 2, Figs. 1 and 2). The most potent hydroxamic acids were trichostatin, CUDC-907, AL179-3b and AR-42: approximately 10-fold less potent than cyromazine, and approximately 50–100–fold less potent than diflubenzuron and dicyclanil. Also showing marked activity ($IC_{50} < 100$ μg/assay) were the thioester compound KD5170, the disulfide compound Psammaplin A (which is a prodrug that forms a thiolate much like romidepsin), and the cyclic tetrapeptide apicidin. Many of the compounds, including 13 of the hydroxamic acids, the two fatty acids (valproic acid and AN-9), and the single sesquiterpene lactone (parthenolide) showed little or no insecticidal activity ($IC_{50} > 1000$ μg/assay).

Comparisons between the larval weight gain and pupation IC_{50} for the commercial insecticides showed that the two values were within 2-fold of each other. For 7 of the 8 most active HDACi (larval $IC_{50} < 100$ μg/assay, Fig. 2), the variation between the larval and pupation IC_{50} values was also within a 2-fold range. The two values were approximately equal for CUDC-907 and AR-42, while within 2-fold for trichostatin, AL1179-3b, romidepsin and KD5170. On the other hand, the pupation IC_{50} for apicidin was 6-fold higher than for larval weight gain.

The HDACi were also investigated for inhibition of HDAC activity in nuclear extracts from blowfly eggs (Table 3), with representative dose-response curves shown in Fig. 3 (some of the compounds shown in Tables 1 and 2 were not examined in nuclear extract assays as insufficient material was available). As with the insecticidal assays, romidepsin was the most potent inhibitor of HDAC activity. This compound was approximately 600-fold more potent than the second most-active compound, quisinostat, and about 1000-fold more potent than trichostatin. The hydroxamic acids that were the most active in the blowfly larval bioassay were among the most potent enzyme inhibitors (IC_{50} 0.016–0.212 μM for trichostatin, CUDC-907 and AR-42). A number of hydroxamic acids that were significant HDAC enzyme inhibitors in the nuclear extracts ($IC_{50} < 0.3$ μM) had low potency in the larval bioassay (e.g. panobinostat, givinostat, belinostat: larval IC_{50} 295, 477, and 740 μg/assay, respectively). Among the other compounds highlighted above for their insecticidal activity (from Fig. 2), all showed significant potency in inhibiting the HDAC enzyme activity of the nuclear extract (all $IC_{50} < 1$ μM).

The relationship between larval bioassay IC_{50} and nuclear extract HDAC inhibition IC_{50} is shown in Fig. 4 (Fig. 4A shows whole data set, Fig. 4B shows data points with extract HDAC inhibition $IC_{50} < 2.0$ μM only). Analysis of the whole data set (Fig. 4A), revealed that the two assay parameters were significantly correlated (Spearman correlation coefficients shown on Figure panels). Despite this, some differences between the two measurements were apparent, with larval weight IC_{50} values of 1000 ($n = 14$) corresponding to a range of nuclear extract activities from 0.032 μM (CUDC-101) to > 100 μM (six compounds). Importantly, low larval weight IC_{50} values (<100 μg/assay) did not occur alongside high nuclear extract IC_{50}. Fig. 4B illustrates this, with the most active insecticidal compounds all being potent inhibitors of HDAC activity in blowfly nuclear extracts ($IC_{50} < 0.5$ μM).

We also examined the relationship between published IC_{50} values for inhibition of human HDAC enzymes by the HDACi used in this study with their activity in inhibiting blowfly larval development. The analysis was restricted to just the human HDACs that corresponded to the Class I and Class II HDAC enzymes present in the blowfly, namely HDAC1 and 3 (Class I) and HDAC4 and 6 (Class II). The published data on the inhibition of human HDAC11 (corresponding to the other HDAC present in the blowfly) was not extensive enough with respect to the HDACi examined in the present study (see Supplementary Table 1) to allow for a separate analysis of this Class IV HDAC. The relationship between the blowfly bioassay data for each HDACi and the reported enzyme inhibition IC_{50} values against the Class I and II human HDAC enzymes are shown in Fig. 5. The two parameters were significantly correlated for the Class I enzymes, but not for the Class II enzymes. However, even though a significant correlation existed for Class I enzymes across the whole data set, a number of compounds that were potent inhibitors of the human Class I enzymes showed no insecticidal activity ($IC_{50} > 1000$ μg/assay). Similarly, some potent human Class II HDAC inhibitors showed no insecticidal activity.

Table 2
Effects of HDACi and commercial insecticides on the development of blowfly larvae.

Drug group	Compound	Blowfly bioassay			
		Weight gain in first 24 h		Pupation	
		IC$_{50}$ (μg/assay)	95% CI	IC$_{50}$ (μg/assay)	95% CI
1) HDAC inhibitors					
Hydroxamic acids	Trichostatin	10.4	5.3–20.4	20.6	16.0–26.6
	CUDC-907	12.2	6.1–24.5	13.8	7.5–25.5
	AL1179-3b	13.9	7.3–26.2	20.0	14.6–27.3
	AR-42	34.0	26.9–43.7	28.0	20.1–38.3
	Quisinostat	100	39–260	274	149–501
	PG50	101	26–388	>200	
	Nexturostat	137	68–279	>1000	
	AL1179-84	254	79–816	918	619-1360
	Panobinostat	295	162–539	393	173–895
	Pracinostat	302	110–834	>1000	
	SBHA	356	215–588	550	405–747
	AL-1179-85	380	90-1607	863	746-1000
	SAHA	434	247–763	>1000	
	Givinostat	477	157-1444	>1000	
	M344	490	294–804	890	632-1257
	Resminostat	556	258-1200	>1000	
	Belinostat	740	426-1294	>1000	
	Naphthohydro. acid	778	335-1810	>1000	
	Droxinostat	>1000		>1000	
	CAY10603	>1000		>1000	
	VAHA	>1000		>1000	
	MC-1568	>1000		>1000	
	ABHA	>1000		>1000	
	NW58	>1000		>1000	
	Tubacin	>1000		>1000	
	HPOB	>1000		>1000	
	BRD73954	>1000		>1000	
	CUDC-101	>1000		>1000	
	Rocilinostat	>1000		>1000	
	Tubastatin A	>1000		>1000	
	PCI-34051	>1000		>1000	
Cyclic depsipeptide	Romidepsin	0.124	0.103–0.149	0.196	0.102–0.374
Benzamides	Entinostat	680	475–974	640	200-2056
	Mocetinostat	>1000		>1000	
Thioester	KD5170	40.6	20.6–79.9	75.3	52.6–107.9
Disulfide	Psammaplin A	56.3	22.9–138.3	93.4	55.1–158.1
Thiolate	TCS HDAC620b	284	171–470	>1000	
Cyclic tetrapeptide	Apicidin	83.3	51.5–134.5	489	218-1097
Fatty acids	Valproic acid	>1000		>1000	
	AN-9	>1000		>1000	
Sesquiterpene lactone	Parthenolide	>1000		>1000	
2) Commercial blowfly insecticides					
Pyrimidine	Dicyclanil	0.115	0.0160–0.829	0.0634	0.0519–0.0776
Diamino-triazine	Cyromazine	1.27	0.673–2.40	1.54	0.600–3.96
Benzoyl phenyl urea	Diflubenzuron	0.230	0.133–0.400	0.119	0.0941–0.151

4. Discussion

The present study has examined the ability of a number of known HDACi to inhibit the growth and development of blowfly larvae, and correlated this effect with their ability to inhibit the HDAC activity of nuclear extracts prepared from blowfly eggs. There was a significant correlation, suggesting that their insecticidal activity was likely due to the inhibition of blowfly HDAC enzymes. Romidepsin was a very potent inhibitor of both blowfly larval growth and blowfly HDAC activity, the potency being equivalent to or greater than commercial blowfly insecticides. In addition, we have shown that a number of other HDACi have significant insecticidal activity against blowfly larvae, including hydroxamic acids (Trichostatin, CUDC-907, AL1179-3b, AR-42), a thioester (KD5170), a disulphide (Psammaplin A) and a cyclic tetrapeptide with a zinc-binding ketone (Apicidin).

While these HDACi validate the concept of a potentially valuable new target for insecticides, we are not advocating the use of the particular compounds reported herein as commercial insecticides. They would be too expensive to be economically viable for any livestock or agronomic production setting. Moreover, most of the more potent HDACi described are also potent inhibitors of human HDACi (IC$_{50}$ nM - μM) and might prove cytotoxic in sheep and unacceptable in terms of human consumption of sheepmeat. Hence, while our demonstration of the potent insecticidal activity of a number of HDACi helps to prove the concept that HDACi may be effective insecticides, issues associated with cost of production and target pest selectivity need to be solved next.

Romidepsin is a prodrug that is first activated by reduction of its disulfide to the free thiol that can then bind to the catalytic Zn^{2+} in HDAC enzymes. Thiols or thiolates have a much lower binding affinity for Zn^{2+} than hydroxamic acids. The higher potency of romidepsin involves either a highly complementary fit of the conformationally constrained cyclic depsipeptide component of romidepsin with the enzyme, or higher metabolic stability than the hydroxamates. Apicidin is another compound with significant

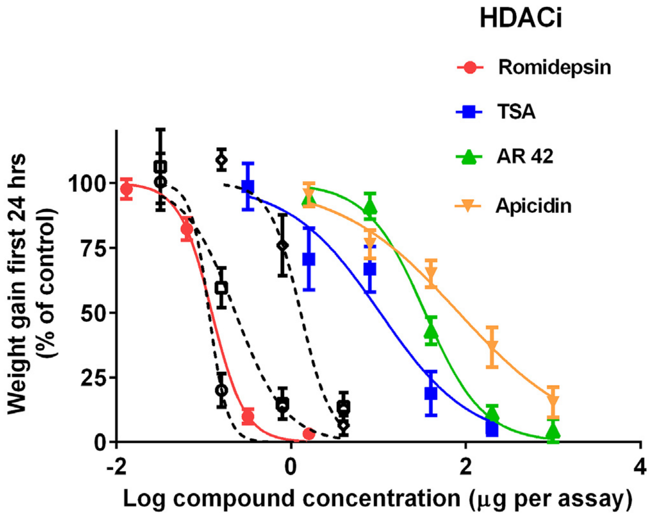

Fig. 1. Effects of HDACi (coloured solid symbols and lines; named in key) and commercial insecticides (open symbols dashed lines; cyromazine ◊, diflubenzuron □, dicyclanil ○) on the growth of blowfly larvae. Each data point represents mean ± SE, n = 2 assays at each compound concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

insecticidal activity (Table 2) which also has a rigid cyclic tetrapeptide component that adds affinity to the relatively weak interaction between its ketone component and zinc. Interestingly, Engel et al. (2015) found that romidepsin inhibited the growth of asexual stage *Plasmodium falciparum* (IC_{50} 0.1 μM), the bloodstream form *Trypanosoma brucei* parasites (IC_{50} 0.035 μM), and was a potent inhibitor of HDAC enzyme activity in *P. falciparum* nuclear extracts (IC_{50} 0.9 nM).

In contrast, most hydroxamic acid based inhibitors derive their affinity from zinc chelation which sometimes compensates for a suboptimal fit between the remaining features of the inhibitor and the enzyme active site. The 31 hydroxamic acids examined here have considerable structural diversity and are mostly potent inhibitors of human HDACs. They show quite a range of inhibitory potencies against blowfly larval growth over two log units (Table 2). Most of the hydroxamate-based inhibitors were derived from 4-aminopyrimidine or 4-aminobenzene hydroxamic acids, which confer an extended linear shape to the fragment projecting towards Zn^{2+} in the enzyme. Trichostatin has a similarly rigid linear structure due to its highly conjugated olefin components. Other active inhibitors with a linear structure due to an aromatic group in conjugation with a double bond and hydroxamate are the cinnamic acid hydroxamates, panobinostat & pracinostat. The potent suberoylhydroxamates (AL1179-3b & PG50) have a more flexible linear zinc-binding extension like the similarly flexible but simpler parent compound SAHA, but exhibit superior activity attributed to their branched capping group that likely makes multiple interactions with the enzyme. PG50 was developed as a selective inhibitor of human HDAC6 (Gupta et al., 2010), however it seems to be a class I HDACi in the blowfly possibly suggesting its capping groups are too small to influence selectivity as the other hydroxamate inhibitors known to specifically inhibit human HDAC6 (tubacin & tubastatin A) were inactive in the blowfly bioassay. The reasons why other hydroxamates were inactive is not clear, but they do show how selectivity between highly homologous enzymes can be achieved, in this case away from blowfly and towards human. In principle this trend might be reversed with new compounds. Clues derived from the capping cyclic peptide groups away from the zinc-binding moieties of romidepsin and apicidin may steer the development of new compounds with greater potency and selectivity for the target enzyme to make better and safer insecticides.

Two aspects of the time course of insecticidal effects are important for blowfly control. Firstly, effective insecticides must kill, or inhibit the growth of early stage larvae before they can damage the host. Secondly, where the initial effects are inhibitory rather than lethal, they must persist over at least several days and then kill the larva to prevent it recovering and developing to damage the host. A comparison of the two bioassay IC_{50} values is informative with respect to these time course considerations. The commercial insecticides show a pupation IC_{50} that is similar (within two fold) to the 24 h weight gain IC_{50}, consistent with the larvae not recovering from an initial growth inhibition phase. This was also observed for seven of the eight HDACi highlighted in Fig. 2. Apicidin on the other hand showed a pupation IC_{50} value almost 6-fold greater than the weight gain IC_{50}, indicating some recovery of larvae after the initial inhibitory effects on growth.

A number of compounds showed potent inhibition of the nuclear extract HDAC activity, but only low or no activity in the larval bioassay (for example: nuclear enzyme assay CAY10603 IC_{50} 0.165 μM, CUDC-101 0.0317 μM *vs* larval bioassay IC_{50} > 1000 μg/assay). This is likely due to poor uptake or low stability of the compounds in the larval assay. There are likely to be differences between the various compounds examined in terms of uptake across the larval cuticle (trans-cuticular uptake) and across the intestinal membranes (following ingestion), as well as access to the cellular target following uptake. Some of the compounds are likely to be metabolised to a greater degree than others by the blowfly xenobiotic-detoxification systems, which include esterases (Campbell et al., 1997), cytochromes P450 (Kotze, 1993) and glutathione transferases (Kotze and Rose, 1987).

Potency against human class I HDAC enzymes generally correlated with insecticidal activity, but some potent inhibitors of human Class I HDAC (IC_{50} < 0.10 μM) showed no insecticidal activity. This may be due to factors associated with uptake and stability of the compounds in the bioassay, as well as differences in the intrinsic level of interaction of the compounds with the human enzymes compared to the equivalent blowfly HDAC enzymes. Kotze et al. (2015) described some differences in the amino acid residues between the human and blowfly Class I HDACs, with catalytic domain amino acids showing 86% and 73% identities between human and blowfly HDAC1 and 3, respectively. The relationship between inhibitory effects of HDACi on human Class II HDACs and their insecticidal activity was poor, with no significant correlation between the two parameters. The catalytic domain amino acids differ to a much greater extent between the human Class II HDACs and their blowfly equivalents compared to the Class I comparisons, with % identities of 61%, 47% and 50% for HDAC 4 and the two catalytic domains of HDAC6, respectively, between the human and blowfly (Kotze et al., 2015). Hence, HDACi of human and blowfly Class II enzymes may show a lower level of relatedness than among inhibitors of Class I enzymes from the two species. The lack of correlation for Class II HDACs may be favourable for potential identification of more insect-specific HDACi that interact specifically with the blowfly Class II enzymes, while showing less inhibition of the human Class II enzymes. However, more information on the different roles played by the blowfly Class I and II HDAC enzymes is required before a preferred target HDAC Class or individual enzyme can be determined. Foglietti et al. (2006) found that RNAi –mediated silencing of *Drosophila* HDACs 1 and 3 resulted in inhibitory effects on growth curves for *Drosophila* Schneider (S2) cell lines, whereas silencing of HDACs 4, 6 and 11 did not inhibit cell growth, suggesting more important roles for the two Class I enzymes in cell viability. Du et al. (2010) reported that *Drosophila* HDAC6 loss-of-function mutant flies were viable and fertile,

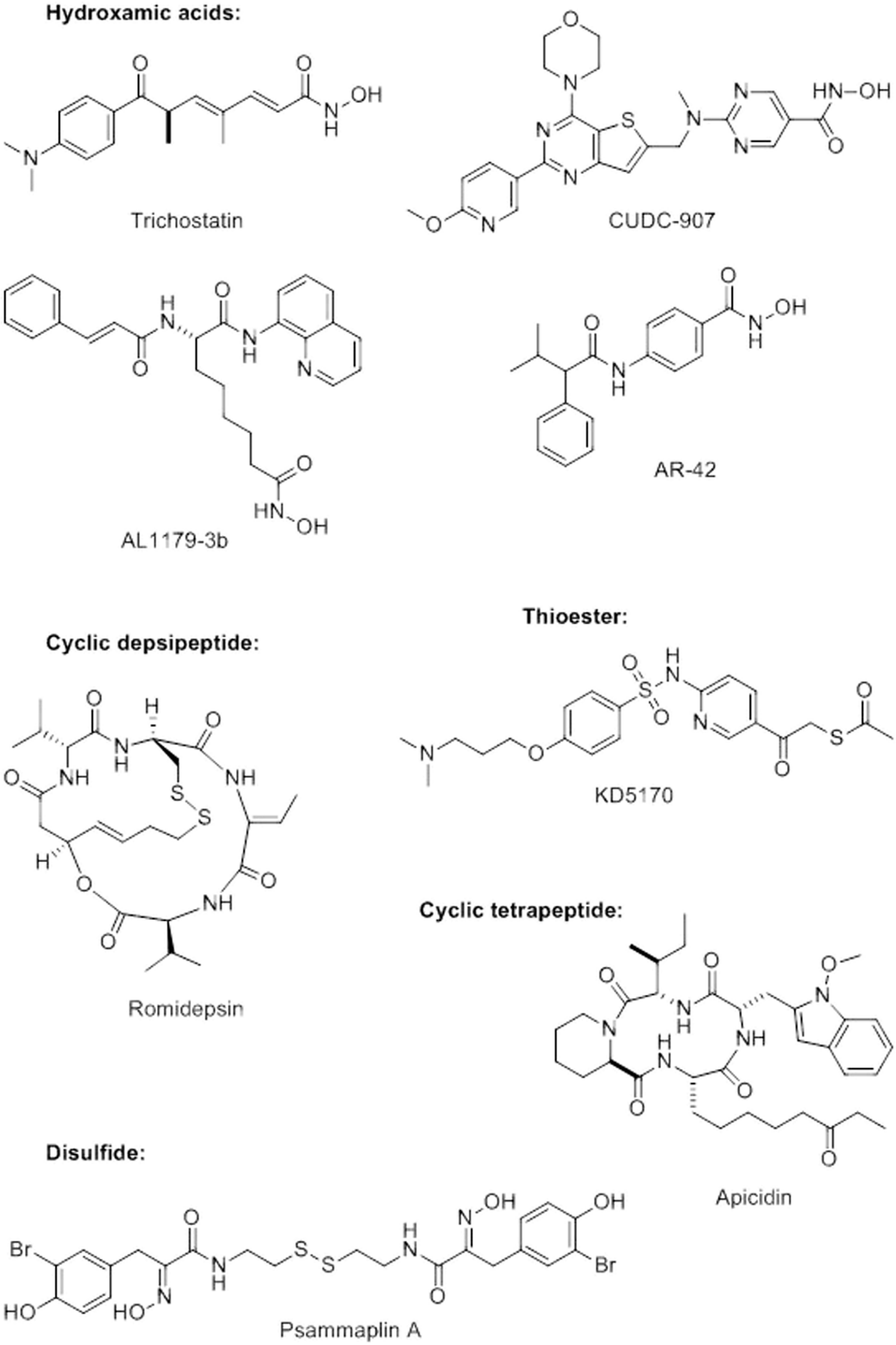

Fig. 2. Structures of HDACi with the most potent inhibition of blowfly larval development (larval weight gain $IC_{50} < 100$ μg/assay).

Table 3
Effects of HDACi on HDAC activity of nuclear extracts from blowfly eggs.

Drug group	Compound	Nuclear extract assay	
		IC_{50} (μM)	95% CI
Hydroxamic acids	Trichostatin	0.016	0.011–0.022
	CUDC-907	0.11	0.08–0.17
	AR-42	0.21	0.18–0.26
	Quisinostat	0.009	0.003–0.022
	Nexturostat	5.1	3.2–8.3
	Panobinostat	0.017	0.012–0.025
	Pracinostat	0.69	0.58–0.82
	SBHA	9.9	6.5–15.2
	SAHA	0.39	0.30–0.50
	Givinostat	0.19	0.15–0.24
	M344	0.58	0.41–0.81
	Resminostat	1.71	1.27–2.30
	Belinostat	0.27	0.19–0.36
	Naphthohydro. acid	83	54–128
	Droxinostat	49	40–59
	CAY10603	0.17	0.10–0.27
	VAHA	>100	
	ABHA	2.6	1.5–4.6
	Tubacin	26	18–37
	HPOB	17	13–20
	BRD73954	>100	
	CUDC-101	0.032	0.014–0.070
	Rocilinostat	2.0	1.6–2.4
	Tubastatin A	71	37–133
	PCI-34051	>100	
Cyclic depsipeptide	Romidepsin	0.000014	0.00001–0.00002
Benzamides	Entinostat	15	5–46
	Mocetinostat	>100	
Thioester	KD5170	0.41	0.32–0.50
Disulfide	Psammaplin A	0.015	0.007–0.032
Thiolate	TCS HDAC620b	>100	
Cyclic tetrapeptide	Apicidin	0.72	0.45–1.14
Fatty acid	Valproic acid	>100	
Sesquiterpene lactone	Parthenolide	>100	

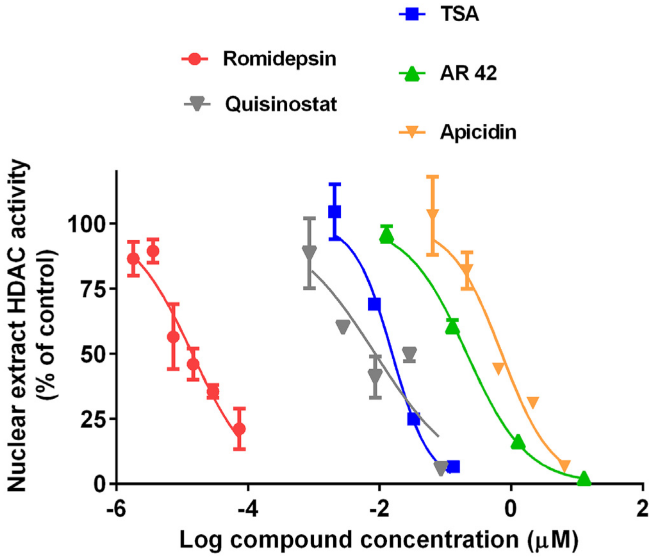

Fig. 3. Effects of HDACi (named in key) on HDAC activity of nuclear extracts prepared from blowfly eggs. Each data point represents mean ± SE, n = 2 assays at each compound concentration.

suggesting that this enzyme may not be essential for the development of this fly species.

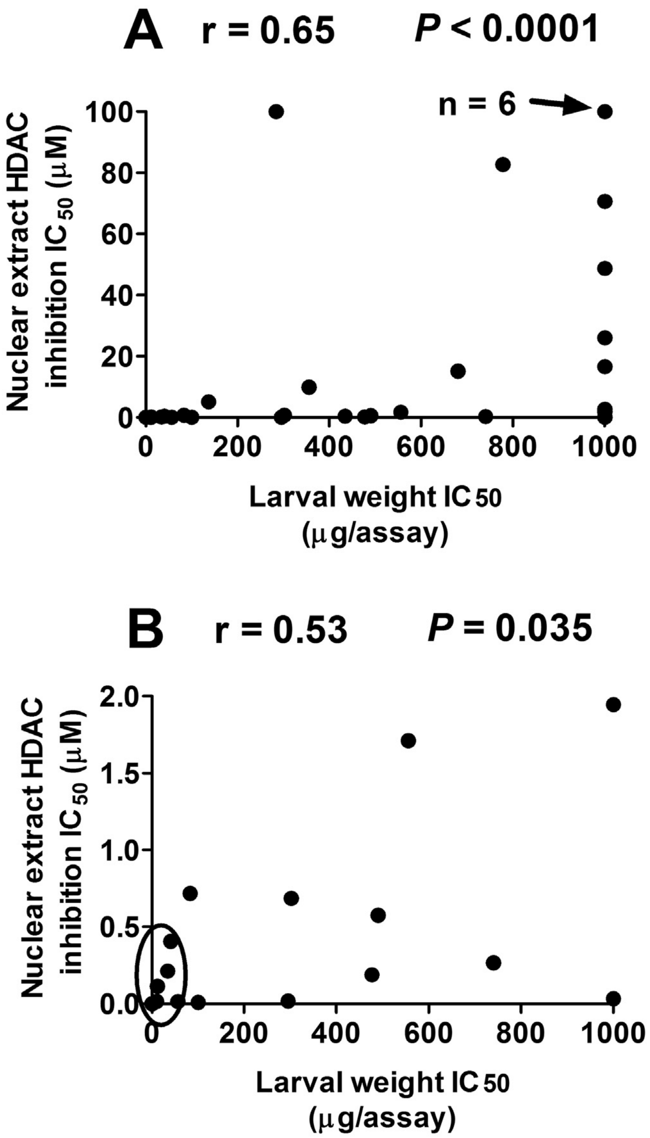

Fig. 4. Relationship for HDACi in inhibiting blowfly larval development (larval weight gain IC_{50}) versus blowfly HDAC activity (nuclear extract IC_{50}). A: whole data set (n = 34), with Spearman correlation coefficient and *P* value; data points at 100 μM for nuclear extract inhibition and/or 1000 μg/assay for larval weight were measured as > 100 and > 1000, respectively. B: Only the most potent inhibitors of HDAC activity (IC_{50} < 2.0 μM, n = 16), with Spearman correlation coefficient and *P* value; circled data points are for romidepsin, TSA, CUDC-907, AR 42, KD5170 and Psammaplin A.

In conclusion, the present study shows that HDACi from various chemical groups can substantially inhibit the development of blowfly larvae. In particular, romidepsin was at least equipotent with the major commercial blowfly insecticides, supporting the concept of inhibiting blowfly HDAC enzymes to produce new insecticides for preventing infection by sheep blowfly, and to potentially control other insects. There is a great deal of interest currently in developing HDAC inhibitors for use in chemotherapy against other human parasitic disease – malaria, toxoplasmosis, trypanosmiasis, schistosomiasis and leishmaniasis (Andrews et al., 2012a,b, 2014; Kelly et al., 2012; Hansen et al., 2014; Engel et al., 2015; Marek et al., 2015). A focus of these studies is the identification of HDACi that show selectivity for the parasite HDAC enzymes over the human enzymes. Similarly, further work on developing HDAC inhibitors as potent insecticides could focus on identifying insect-specific inhibitors, but at the very least should focus on producing HDACi that are cheap to manufacture and market as prospective insecticides.

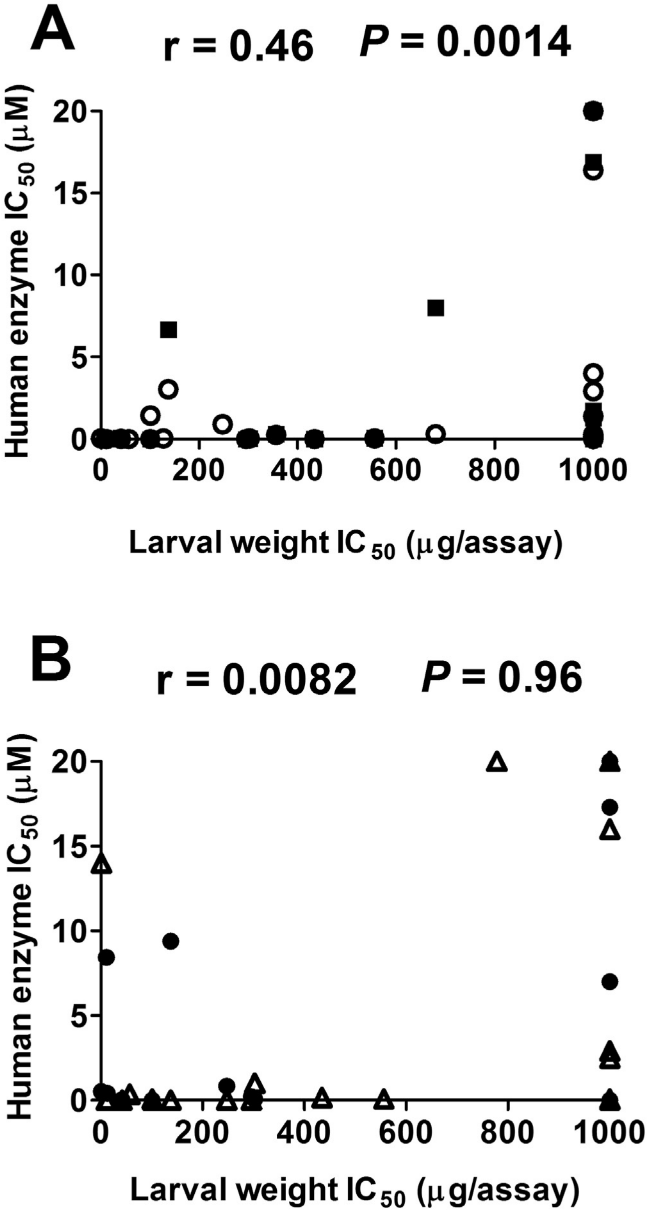

Fig. 5. Relationship for HDACi in inhibiting larval development (larval weight gain IC$_{50}$) versus human Class I enzymes (HDAC1 ○, HDAC3 ■) (A) (n = 45), and human Class II enzymes (HDAC4 ●, and HDAC6 Δ) (B) (n = 37). Spearman correlation coefficients and *P* values shown. Data points at 20 μM for human enzyme inhibition and/or 1000 μg/assay for larval weight were measured as > 20 and > 1000, respectively.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2017.01.001.

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