Efficacy of the cyclooctadepsipeptide PF1022A against *Heligmosomoides bakeri in vitro* and *in vivo*

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SUMMARY

The cyclooctadepsipeptide PF1022A derived from the fungus, *Mycelia sterilia*, is characterized by a broad spectrum of activity against different parasitic gastrointestinal nematodes of livestock. In the present work the anthelmintic activity of PF1022A against *Heligmosomoides bakeri*, a widely used laboratory model was studied. Albendazole, ivermectin and levamisole served as reference. *In vitro*, PF1022A showed low activity on embryonation but significantly inhibited egg hatch (10 and 100 μg/ml), whereas albendazole (10 and 100 μg/ml) revealed statistically significant inhibitions of both embryonation and egg hatch. PF1022A (1–100 μg/ml) completely inhibited larval movement at most examination points. Comparable significant anthelmintic activity on the larval stages of *H. bakeri* was observed with levamisole (48–100%), while slightly lower activities were observed with ivermectin (20–92%) and albendazole (0–87%) at 1–100 μg/ml. PF1022A and levamisole significantly inhibited motility and egg release of adult worms, while albendazole and ivermectin failed to demonstrate activity. Significant worm burden reductions were achieved with PF1022A, levamisole and ivermectin *in vivo*. For example, at 0·125 mg/kg PF1022A a worm burden reduction of 91·8% was observed. The use of drug combinations did not further enhance the *in vitro* and *in vivo* activity of PF1022A. In conclusion, further investigations are warranted with PF1022A, as the drug is characterized by significant larvicidal and nematocidal activity *in vitro* and *in vivo*.

Key words: cyclooctadepsipeptides, PF1022A, albendazole, levamisole, ivermectin, *Heligmosomoides bakeri*.

INTRODUCTION

Soil-transmitted helminthiases are neglected tropical diseases caused by *Ascaris lumbricoides* (roundworm), *Trichuris trichuria* (whipworm), *Strongyloides stercoralis* (threadworm) and the hookworms, *Ancylostoma duodenale* and *Necator americanus*. Soil-transmitted helminth infections affect more than a quarter of the human population, mostly in low-income countries in tropical and subtropical regions of the world (Bethony *et al*. 2006; Chan, 1997; Hotez *et al*. 2007). It is difficult to estimate the amount of morbidity and mortality caused by intestinal nematodes. In 1997 it was estimated that intestinal nematode infections cause the loss of 39 million disability-adjusted life years (DALYs) of which *A. lumbricoides* infections account for 10·5 million, *T. trichuria* infections for 6·4 million, and infections with hookworm for 22·1 million (Chan, 1997). In the framework of the ongoing new Global Burden of Disease study the global distribution and disease burden of intestinal nematodiasis is currently being evaluated and re-analysed (Brooker, 2010).

Soil-transmitted helminthiases control programmes aim to reduce morbidity through regular treatment; however, only a handful of drugs are available, albendazole, mebendazole, levamisole and pyrantel pamoate (Keiser and Utzinger, 2010). Drug resistance is a main concern under such high selective pressure. Indeed, in veterinary medicine anthelmintic resistance has established at relatively low treatment frequencies (Coles, 1999; Coles and Klei, 1995). In addition, the currently used drugs have several shortcomings: for example, none of the drugs is highly effective against *T. trichuria* when administered at single doses (Keiser and Utzinger, 2008). Hence, it is imperative that new anthelmintic drugs are developed.

The cyclooctadepsipeptide PF1022A, is a natural compound produced by fermentation of the fungus *Mycelia sterilia* (Sasaki *et al*. 1992). Anthelmintic activities of PF1022A and emodepside, a semi-synthetic derivative, have been demonstrated in several *in vitro* and *in vivo* studies against various nematodes. For example, the larval and adult stages of *Heterakis spumosa* were highly susceptible to PF1022A (Harder *et al*. 1997). In a jird model, *Haemonchus contortus, Trichostrongylus colubriformis,*

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and *Ostertagia ostertagi* were susceptible to oral PF1022A, with declined efficacies noted when the drug was administered parenterally (Conder *et al*. 1995). PF1022A was also highly active against *Toxocara canis* and *T. cati* in dogs (Fukashe *et al*. 1990). Broad-spectrum anthelmintic activity has also been demonstrated with emodepside against the adult stages of *Heligmosomoides polygyrus, Strongyloides ratti* and *Nippostrongylus brasiliensis* (Harder and von Samson-Himmelstjerna, 2002). Most importantly, cyclooctadepsipeptides are active against resistant isolates (Harder and von Samson-Himmelstjerna, 2002), which has been explained by their unique mechanism of actions, the simultaneous stimulation of the GABAergic neurotransmission and inhibition of the cholinergic systems (Harder *et al*. 2005; Terada, 1992). In more detail, emodepside has been demonstrated to act through a SLO-1 dependent pathway, by activating a calcium-activated potassium channel (Guest *et al*. 2007).

A combination of emodepside and praziquantel (Profender®, Bayer AG, Leverkusen, Germany) is marketed for use in companion-animals (Mehlhorn *et al*. 2005; Schroeder *et al*. 2009).

The aim of this study was to further strengthen our knowledge on the nematocidal properties of PF1022A. We investigated the *in vitro* and *in vivo* efficacy of PF1022A against the trichostrongylid nematode *Heligmosomoides bakeri*, formerly known as *H. polygyrus* and *Nematospiroides dubius*. *In vitro* inhibition of egg development, larval and adult motility and egg release were studied following incubation with PF1022A. Albendazole, levamisole and ivermectin were used as reference drugs. In addition, drug combinations of PF1022A plus ivermectin, levamisole or albendazole were studied *in vitro*. Finally, the dose-response relationships of PF1022A, ivermectin, levamisole and albendazole as well as combinations of PF1022A with these drugs were studied *in vivo*.

MATERIALS AND METHODS

Drugs

PF1022A was obtained from Bayer AG, Monheim. Albendazole, levamisole, and ivermectin were purchased from Sigma Aldrich (Buchs, Switzerland). Stock solutions of PF1022A, albendazole, levamisole and ivermectin were prepared by dissolving 10 mg of each drug in 1 ml of 99·9% dimethylsulfoxide ((DMSO), Fluka Analytical, Sigma-Aldrich).

Parasite

The infective third-stage larvae (L3) of *H. bakeri* used in our work were obtained from the Swiss Federal Institute of Technology, Zurich. The *H. bakeri* life cycle was maintained in female NMRI mice in our laboratories at the Swiss Tropical and Public Health Institute (Basel, Switzerland), following national and cantonal regulations on animal welfare (Permit no. 2070).

L3 larvae were cultured from faeces of infected mice using the coproculture technique described by Burren (1980). Briefly, collected faeces were pooled together, mixed with distilled water and filtered. The filtrate was centrifuged and the supernatant discarded. The sieved faeces (sediment) was then thinly smeared onto 3 moistened Whatman® 40 filter papers, and incubated in a Petri dish for 7 days at 25 °C. After the harvest of L3 larvae, the larval suspension was adjusted to a larval concentration of 30 L3 larvae per 20 μl. The L3 larvae were suspended in distilled water and stored at 4 °C. For the larval motility assays, L3 larvae less than 72 h old were used.

The first- (L1) and second-stage larvae (L2) used for the larval motility assay were cultured directly from eggs recovered from mice faeces. The eggs were cultured in a Petri dish containing distilled water for 48 h. Harvested L1/L2 larvae (30 L1/L2 larvae per 20 μl) were used immediately to prevent their development into L3 larvae. Both larval suspensions contained 100 U/ml of penicillin, 100 μg/ml streptomycin (Gibco) and 12·5 μg/ml of amphotericin B (Sigma).

Preparation of eggs

Faecal pellets of *H. bakeri*-infected mice were collected over a period of 3 h using wire-bottomed cages lined with moist paper towel. The faeces were soaked (1:10) in 0·9% NaCl for 30 min and vortexed. The faecal mixture was then filtered through a tea strainer (0·1 mm aperture) and the filtrate was centrifuged at 500 ***g*** for 5 min in a 50 ml Falcon tube (Sarstedt). The sediment was resuspended in a 25% glucose solution (α-D-glucose 96%, Aldrich Chemistry). A layer of 1 ml of distilled water was placed on top of the faecal/glucose solution and centrifuged at 500 ***g*** for 5 min at 15 °C. The eggs were recovered from the interface between the distilled water and the glucose and rinsed with distilled water. The recovered eggs were suspended in distilled water containing antibiotics and fungicide.

Egg hatch test

Thirty unembryonated eggs (20 μl) were placed in 48-well plates containing RPMI 1640 (470 μl) and 10 μl of the test drugs (1 to 100 μg/ml). The plates were incubated at 25 ± 1 °C for 40 h. Control wells contained 1% DMSO. The egg hatch test was carried out as described by Fonseca-Salamanca and coworkers (Fonseca-Salamanca *et al*. 2003), with minor modifications. The ovicidal effect of drugs at each concentration was quantified by counting

embryonated and unembryonated eggs at 24 h, and the numbers of L1/L2 larvae and unhatched eggs in each well at the end of incubation. These data were compared to the control wells (after correcting for undeveloped eggs). Data-points consist of 3 independent assays, with duplicate assay wells, for each drug concentration. Between 4 and 6 control wells were included per assay.

Larval motility assay

L1/L2 or L3 larvae were placed in wells (30 per well) containing RPMI and increasing concentrations (0·1–100 μg/ml) of the test drugs. Control wells contained 1% DMSO. Larval motility was observed under an inverted microscope (X100) and anthelmintic activity was recorded at 2, 24 and 48 h (L1/L2 assay) and at 2, 24, 48 and 72 h (L3 assay) after incubation at 25 °C, respectively. Worms were classified as either, 0 – inhibited, not moving/paralysed or 1 – alive, moving/twitching.

In preliminary assays, untreated L1/L2 control larvae were observed to be actively motile at the 2 and 24 h time-points but not at the 48 h time-point. Therefore, an external stimulation of wells at 48 h with hot water (Satou *et al*. 2001) was used to evoke motility in the L1/L2 motility assay. Briefly, at the 48 h time-point 500 μl of hot water (80 °C) was added to each well and the larval motility observed after 20–30 sec. For the L3 assay, wells were stimulated at all 4 time-points. All externally stimulated wells were excluded from further reading.

Percentage survival in each well was calculated by counting treated motile and immotile larvae and comparing them to motile larvae in control wells. Three independent experiments were performed, with a range of 4 to 8 assay wells for each drug concentration and control.

L3 larval motility assay with drug combinations

The following 4 drug combinations were studied: PF1022A (0·1 μg/ml) plus albendazole (1 μg/ml), ivermectin (1 μg/ml) or levamisole (0·1 μg/ml) and PF1022A (0·05 μg/ml) plus ivermectin (0·5 μg/ml). A 24-well plate assay (1000 μl) which contained the drug combinations, 780 μl of RPMI, 20 μl of L3 larval suspension was incubated at 25 °C and observed 72 h post-incubation. The anthelmintic activities of combination treatments were assessed in 4 independent experiments, with triplicate assay wells for each drug combination.

Adult motility assay

Six female NMRI mice were infected with 150 infective L3 larvae by gavage. At 11 days post-infection mice were killed with CO_2 and the small intestine of each mouse was removed, opened longitudinally and incubated in pre-warmed saline. The adult worms were retrieved by picking, and placed in Petri dishes that contained supplemented RPMI 1640 pre-warmed at 37 °C.

Adult worms were washed with RPMI and 2 females and 2 males were placed into each well of a 48-well plate which contained 0·5 ml phenol-red free RPMI supplemented with 25 mM HEPES, 500 U/ml penicillin, 500 μg/ml streptomycin, 0·6 μg/ml amphotericin B and PF1022A, albendazole, ivermectin, levamisole (100–0·1 μg/ml) or solvent (0·2% DMSO). Each plate was incubated at 37 °C with 5% CO_2 (Innova® CO-48, Newbrunswick Scientific) for 72 h. The anthelmintic activity of tested drugs on adult worms was quantified as described above using an inverted microscope. The adult motility assay was performed once, in triplicate assay wells for each tested drug concentration and control.

Egg release

For the evaluation of the effect of drugs on *H. bakeri* egg release 3 female worms were placed in each well of a 48-well plate that contained supplemented RPMI with different concentrations of albendazole, levamisole, ivermectin and PF1022A. Untreated worms served as controls. After incubation for 24 h at 37 °C in 5% CO_2, adult worms were removed and the culture medium containing eggs was retrieved and each well rinsed with 500 μl of distilled water. The number of eggs released was calculated for each drug and drug concentration by counting 4 aliquots of 20 μl from each well. The percentage reduction was calculated by comparing drug-treated wells to control wells.

In vivo *studies*

Eighty-four female NMRI mice were orally infected with 150 infective L3 larvae. At 11 days post-infection mice (groups of 4) were treated with single oral doses of 0·0625, 0·125, 0·25 or 0·5 mg/kg PF1022A, 0·25, 0·5 or 1 mg/kg ivermectin, 1·25, 2·5, 5 or 10 mg/kg levamisole, and 5 and 10 mg/kg albendazole. Three groups of mice ($n=4$ each) received 0·0625 mg/kg PF1022A combined with 0·125 mg/kg ivermectin, 1·25 mg/kg levamisole or 10 mg/kg albendazole. Four groups of 4 infected but untreated mice served as controls. At 6 days post-treatment mice were killed with CO_2 and the adult worms collected and counted as described above.

Statistical analyses

The proportions of inhibition for egg embryonation, hatching, worm motility and egg release were calculated as a percentage (mean ± standard deviation)

using Microsoft® Excel. The Fisher's exact test was used to assess statistical significance of the different drugs on egg hatch test, larval motility assays (L1/L2 and L3) and adult motility assay at a 5% level of significance, using GraphPad Instat (version 3.10, San Diego, CA, USA). The Kruskal-Wallis (KW) test (Statsdirect statistical software (version 2.4.5, Statsdirect Ltd, Cheshire, UK)) was used to compare the medians of the *H. bakeri* worm counts (control versus each of the 4 drugs at all doses tested). Differences in medians were considered to be significant at a significance level of 0·05. ED_{50} and combination index (CI) values (Chou, 1998) were calculated using the CompuSyn software.

RESULTS

Egg hatch test

The effects of PF1022A, albendazole, ivermectin and levamisole on embryonation and egg hatch are summarized in Table 1. PF1022A, ivermectin and levamisole had no significant effect on egg embryonation, regardless of the concentration tested. Egg hatching was significantly affected by levamisole and PF1022A at the 2 highest concentrations tested (100 and 10 μg/ml). The most effective drug was albendazole, which showed a significant ($P<0·001$) reduction in embryonation and egg hatch of unembryonated eggs of *H. bakeri* regardless of the concentration used.

Table 1. Efficacy of PF1022A, albendazole, ivermectin and levamisole on embryonation and hatching of unembryonated eggs of *Heligmosomoides bakeri*

(Each inhibition (%) represents the mean (standard deviation) of 3 independent experiments, with each drug concentration carried out in duplicate.)

Drug	Concentration (μg/ml)	Inhibition (%) of embryonation at 24 h (S.D.)	Inhibition (%) of hatching at 40 h (S.D.)
Control	—	0	0
PF1022A	100	2·0 (2·0)	4·7 (2·3)
	10	1·0 (1·0)	46·7 (1·5)*
	1	2·0 (2·0)	18·0 (8·5)*
Albendazole	100	100*	100*
	10	99 (1·7)*	100*
	1	25·3 (37·8)*	43·7 (49·2)*
Ivermectin	100	4·7 (5·0)	5·0 (1·7)
	10	1·0 (1·0)	2·7 (0·6)
	1	1·0 (1·0)	4·7 (1·5)
Levamisole	100	0·3 (0·6)	69·7 (10·2)*
	10	1·3 (2·3)	67·0 (5·3)*
	1	4·0 (1·0)	16·3 (4·6)*

* Statistically significant ($P<0·001$).

Larval motility assays

The effects of PF1022A, levamisole, albendazole and ivermectin on the motility of L1/L2 larvae of *H. bakeri* are summarized in Table 2. Incubation with 1–100 μg/ml of PF1022A resulted in a rapid paralysis of L1/L2 larvae at the 2 h time-point, with none of the larvae showing movement ($P<0·001$). High, significant activity ($P<0·001$) was also observed with levamisole (0·1–100 μg/ml). A reversed inhibitory effect (92·0 versus 47·5%) was noted for the highest concentration of levamisole tested (100 μg/ml) at the 2 and 24 h time-points. The reductions of motility calculated for ivermectin (1–100 μg/ml) increased from 29·5–36·5% after 2 h ($P<0·001$) to 79·0–92·0% ($P<0·001$) after 72 h. Albendazole showed the slowest activity on L1/L2 larvae: while low to moderate anthelmintic activities were observed at the 2 h and 24 h time-points (1·5–30·5% inhibition) high activities (63–87%) were observed after 48 h.

Inhibition of L3 larval motility following incubation with PF1022A, albendazole, ivermectin and levamisole are presented in Table 3. In the presence of 1–100 μg/ml of PF1022A, L3 larvae movements were completely inhibited at most examination points. Significant ($P<0·001$) motility reductions (79–100%) of L3 larvae were also observed for levamisole (1–100 μg/ml) at 24–72 h post-incubation. Ivermectin at 1–100 μg/ml significantly ($P<0·001$) inhibited the motility of L3 larvae at the 24 h time-point. However, after 72 h of incubation the inhibitory effect of ivermectin on L3 larval motility had decreased. Albendazole showed weak to moderate inhibitions (13·6–51%) at any of the concentrations tested.

L3 Larval motility assay with drug combinations

In the combination treatment assays (Fig. 1), PF1022A (0·1 μg/ml) plus ivermectin (1 μg/ml) inhibited L3 larvae movement by 99·3% at 72 h. Using half of the concentrations (0·05 μg/ml PF1022A plus 0·5 μg/ml ivermectin), L3 larval motility was inhibited by 76·9% 72 h post-incubation. Low efficacies (65 and 57%) were observed with combination treatments of PF1022A (0·1 μg/ml) plus levamisole (0·1 μg/ml) or albendazole (1 μg/ml), respectively. Analysis based on the Chou-Talalay CI theorem showed that none of the combinations were additive or synergistic but rather revealed antagonistic effects ($CI>1·2$).

Adult motility assay

The activity of PF1022A, ivermectin, albendazole and levamisole against adult *H. bakeri* is presented in Fig. 2. The mean motility rate of adult *H. bakeri* in control wells at 72 h was 93·8%, which was

Table 2. *In vitro* effect of PF1022A, albendazole, ivermectin and levamisole on the motility of L1/L2 larvae of *Heligomosomoides bakeri*

(Inhibition (%) data (mean ± standard deviation) from 3 independent experiments with duplicate assay wells; except for albendazole and ivermectin at 0·1 μg/ml, with data calculated from a single assay.)

			PF1022A				Albendazole				Ivermectin				Levamisole			
Drug		Control	Inhibition of motility (%) (S.D.)															
Concentration (μg/ml)			100	10	1	0·1	100	10	1	0·1	100	10	1	0·1	100	10	1	0·1
Time (h)	2	0	100*	100*	100*	6·5 (0·7)	1·5 (2·1)	3·0 (4·2)	0	NT	36·5 (33·2)*	36·0 (31·1)*	29·5 (29·0)*	NT	92·0 (7·1)*	94·5 (3·5)*	89·5 (0·7)*	11·0 (5·7)*
	24	0	100*	100*	100*	17·5 (2·1)**	15·5 (9·2)	30·5 (27·6)*	16·5 (13·4)**	18·8	36·0 (5·7)*	34·0 (2·8)*	30·0 (15·6)*	10·0	47·5 (13·4)*	98·5 (2·1)*	100*	31·5 (3·5)*
	48	0	100*	100*	100*	57·5 (12·0)	87·0 (2·8)*	63·0 (7·1)	79·0 (5·7)*	18·8	92·0 (11·3)*	85·5 (10·6)*	79·0 (5·7)**	0	98·0 (2·8)*	100*	100*	64·5 (30·4)

* Statistically significant ($P<0·001$) and ** ($P<0·05$). NT: not tested.

Table 3. *In vitro* efficacy of PF1022A, albendazole, ivermectin and levamisole against L3 larvae of *Heligmosomoides bakeri*

(Each data-point represents the mean ((± standard deviation), with 3–8 assay wells from 3 independent trials); except for albendazole and ivermectin at 72 h, which is from a single assay.)

			PF1022A				Albendazole				Ivermectin				Levamisole			
Drug		Control	Inhibition of motility (%) (SD)															
Concentration (μg/ml)			100	10	1	0·1	100	10	1	0·1	100	10	1	0·1	100	10	1	0·1
Time (h)	2	0	100*	84·6 (18·7)*	26·3 (29·0)	26·6 (14·3)	23·3 (7·8)**	23·6 (14·6)**	15·6 (11·3)*	13·6 (7·1)*	85·1 (18·9)*	68·5 (11·6)*	19·6 (9·0)**	19·5 (15·3)**	76·0 (19·6)*	48·3 (26·5)	22·7 (11·1)	19·1 (11·9)**
	24	0	100*	100*	100*	21·0 (9·9)**	33·0 (15·4)*	16·0 (6·6)**	22·0 (6·5)	19·0 (6·5)	74·0 (23·3)*	67·0 (16·8)*	88·0 (3·6)*	29·6 (4·3)	90·0 (17·3)*	91·0 (12·5)*	79·0 (17·1)*	25·5 (12·0)*
	48	0	100*	100*	100*	55·0 (5·7)	43·7 (19·6)*	39·0 (16·5)**	41·0 (14·2)**	23·3 (5·8)	56·7 (5·1)*	65·0 (2·0)*	66·0 (5·1)*	20·0 (9·8)	100*	100*	96·0 (6·7)*	65·0 (4·2)*
	72	0	100*	100*	100*	57·0 (8·5)	51·0	45·0	48·0	21·2 (11·8)	41·0	56·0	64·0**	25·2 (10·9)	100*	100*	100*	96·0 (3·5)*

* Statistically significant ($P<0·001$) and ** ($P<0·05$).

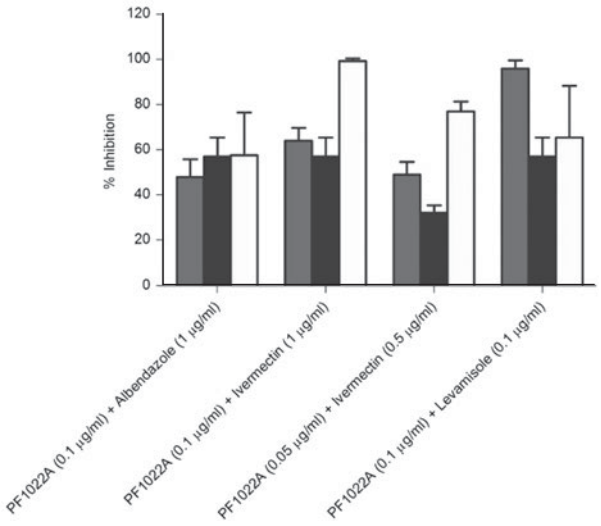

Fig. 1. Efficacy of combination treatment against L3 larvae of *Heligmosomoides bakeri* 72 h post-incubation. The grey bars represent the partner drugs (albendazole, ivermectin or levamisole), the black bars represent PF1022A and the white bars represent drug combinations. Data-points of combination treatment represent 4 independent experiments consisting of triplicate assay wells for each drug concentration. Error bars indicate standard deviation.

comparable to the motility at commencement of the assays. At 72 h post-incubation with 100 μg/ml of the test drugs, PF1022A demonstrated moderate (67·2% ($P<0·05$)) activity against adult worms of *H. bakeri*, levamisole showed a slight inhibition of 12·5%, while no activity was observed with ivermectin and albendazole ($EC_{50}>100$ μg/ml for albendazole and ivermectin). At 10 μg/ml, PF1022A and levamisole reduced movement of adult worms by 78·1% ($P=0·001$) and 100% ($P<0·001$), respectively, at 72 h. Following incubation with 1 μg/ml of drugs, only levamisole demonstrated significant ($P<0·05$ and $<0·001$) activity at all examination points. For PF1022A and levamisole EC_{50} values of 2.7 and 1.3 μg/ml, respectively were calculated.

Egg release

The effect of PF1022A, ivermectin and levamisole on the egg production of female *H. bakeri* worms is shown in Fig. 3. Ivermectin failed to inhibit the egg production at any concentration tested, while albendazole showed only a moderate activity at 100 μg/ml (49·0%). Worms incubated in the presence of PF1022A showed reductions in the number of eggs ranging from 74·9% (10 μg/ml) to 80·9% (100 μg/ml). The highest activities were observed following exposure to levamisole, which reduced the egg release at all concentrations tested. For example, at 10 μg/ml of levamisole, 10 eggs were released within 24 h, which was a 95·2% reduction compared to control.

In vivo *studies*

Monotherapy. Worm burden reductions of 90·4–95·9% were achieved with PF1022A at doses of 0·125 mg/kg and above. At 0·0625 mg/kg PF1022A a low worm burden reduction of 24·5% was observed. The worm burden reductions in PF1022A-treated mice were statistically significant ($KW=8·629$, $p=0·003$). An ED_{50} of 0·07 mg/kg was calculated for PF1022A. Similarly, when ivermectin (0·125 −1 mg/kg) was given to *H. bakeri*-infected mice significant worm burden reductions of 53·7–98·6% (ED_{50} of 0·11 mg/kg) ($KW=11·324$, $p<0·001$) were documented. Levamisole administration (1·25–10 mg/kg) resulted also in high, significant worm burden reductions of 61·5–100% (ED_{50} of 1·05 mg/kg). A moderate, non-significant worm burden reduction was achieved, when a single oral dose of 10 mg/kg albendazole was given to *H. bakeri*-infected mice (ED_{50} of albendazole 9·8 mg/kg (Table 4)).

Combined treatment. Since we were interested to learn whether PF1022A combinations might act additively or synergistically *in vivo* median effective doses of PF1022A (0·0625 mg/kg) were combined with the partner drugs following the constant ratio combination design (Chou, 1998). Combinations of PF1022A and albendazole (10 mg/kg) had no effect on the worm burden and a lower activity than albendazole or PF1022A monotherapy. Moderate worm burden reductions of 59·1 and 66·3% were observed with PF1022A plus ivermectin (0·125 mg/kg) or levamisole (1·25 mg/kg), respectively, which were, however, not significantly higher than the single drugs (Table 4). Since no additive or synergistic effects were observed further doses were not tested for any of the combinations.

DISCUSSION

The use of *in vitro* assays to screen for lead compounds with anthelmintic activity has been pivotal to drug discovery and development for helminth chemotherapy (Keiser, 2010). *In vitro* helminth drug assays are ethical, often simple, of low cost and allow for medium-throughput screening of compounds (Keiser, 2010). In this study, we conducted different *in vitro* assays, such as the egg hatch test, larval motility assays and the adult motility assay including studies on egg production to compare the efficacy of PF1022A against multiple life-cycle stages of the murine parasite *H. bakeri*. Albendazole, ivermectin and levamisole were used as reference drugs in these experiments. *In vivo* studies complemented our work.

PF1022A showed excellent nematocidal properties against the different life-cycle stages of *H. bakeri*. Our finding is in agreement with previous reports, which

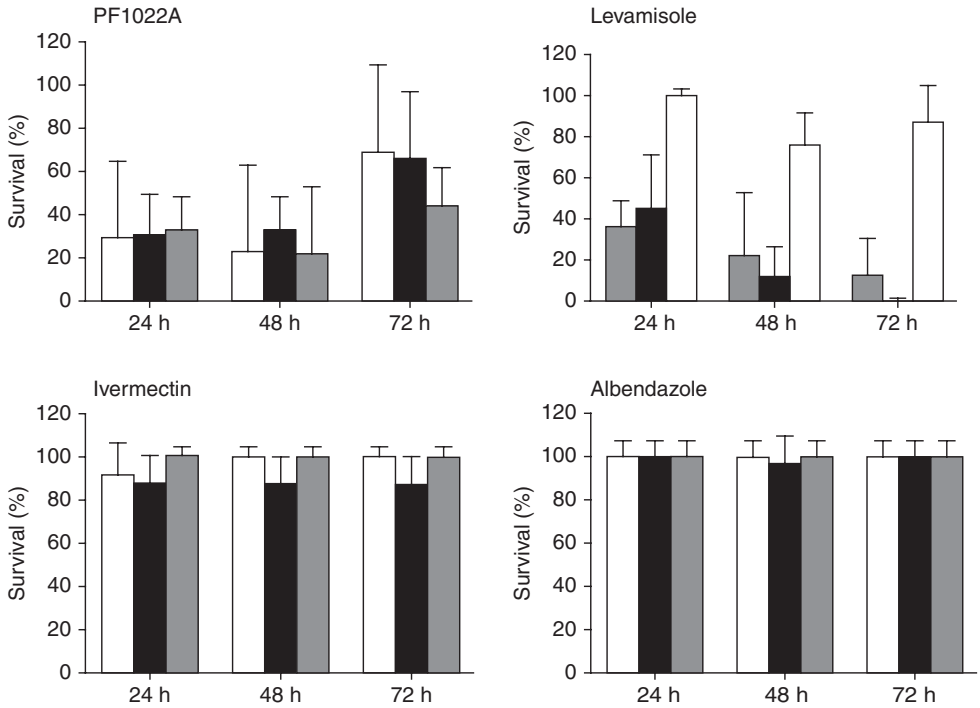

Fig. 2. Effect of PF1022A, ivermectin, levamisole and albendazole against adult worms of *Heligmosomoides bakeri* at 100 μg/ml (white bars), 10 μg/ml (black bars) and 1 μg/ml (grey bars). Data-points derived from a single assay; with 3 replicate wells for each drug concentration and control. Error bars indicate standard deviation.

documented high anthelminthic activities of PF1022A (Harder *et al*. 1997; Harder and von Samson-Himmelstjerna, 2002). PF1022 showed better efficacies than the standard drugs albendazole, ivermectin or levamisole, which is in line with findings documented against adult females of *H. contortus* (Conder, *et al*. 1995).

In the larval motility assays, PF1022A showed the highest activity among all drugs tested. PF1022A was highly effective against both L1/L2 and L3 larvae. Similarly, levamisole produced pronounced effects, while albendazole and ivermectin showed slightly lower activities against L1/L2 and L3 larvae. Interestingly, L1/L2 larvae incubated with levamisole (100 μg/ml) showed no movement at the 2 h time-points, regained movement at the 24 h time-point and became again immotile at 48 h. This phenomenon, also termed 'tachyphlaxy', has been demonstrated with levamisole using L3 larvae of *Ostertagia ostertagi* (Geerts *et al*. 1989) and following incubation of L3 larvae of *Ancylostoma caninum* with pyrantel (Kopp *et al*. 2008). This finding was not observed with *H. bakeri* L3 larvae at the highest concentration of levamisole tested.

The nematocidal activity observed with the test drugs against adult *H. bakeri in vitro* was similar to the findings observed with the free-living stages. PF1022A demonstrated the highest activity against adult worms at concentrations of 100 and 10 μg/ml, while levamisole was the most efficacious drug at 1 μg/ml. The contradictory findings observed with levamisole (inhibition observed with 10 and 1 μg/ml

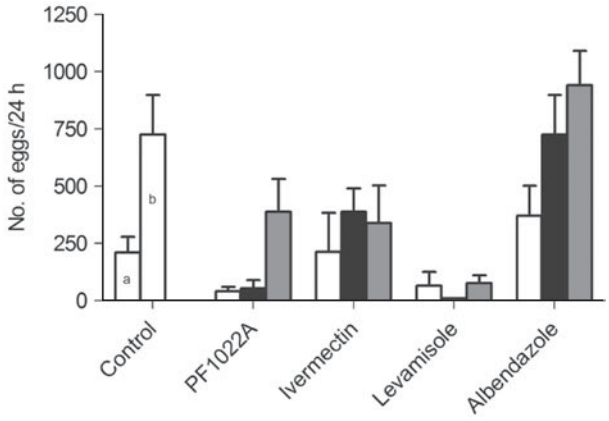

Fig. 3. Efficacy of albendazole, ivermectin, levamisole and PF1022A on the egg release of female *Heligmosomoides bakeri* 24 h post-incubation. Controls (white bars), 100 μg/ml (white bar), 10 μg/ml (black bar) and 1 μg/ml (grey bar); error bars indicate standard deviation. Data-points derived from a single assay with 3 to 10 replicate wells for each drug concentration and control. The activity of ivermectin, levamisole and PF1022A on the egg release was calculated in comparison to control (a), and albendazole was analysed in comparison to control (b).

but no activity observed with 100 μg/ml) might be due to the cholinergic mechanism of action of the drug. A similar result was obtained with tribendimidine; an anthelmintic drug with a related mechanism of action, using the same assay format (unpublished observations). Ivermectin was the least active drug

Table 4. *In vivo* efficacy of PF1022A, ivermectin levamisole and albendazole and combinations of PF1022A and ivermectin, PF1022A and levamisole, and PF1022A and albendazole against adult *Heligmosomoides bakeri*

(S.D., standard deviation, KW: Kruskal Wallis.)

Treatment	Drug	Dose (mg/kg) (single)	Number of animals cured	Mean number of worms (SD)	Total worm burden reduction (%) (95% CI)	ED_{50} (mg/kg)	KW	*P*-value
	Control 1	—	0	18·3 (9·6)	—		—	—
	Control 2	—	0	9·0 (8·4)	—		—	—
	Control 3	—	0	23·8 (18·7)	—		—	—
	Control 4	—	0	10·4 (7·8)	—		—	—
Monotherapy	PF1022A	0·5[a]	2	0·75 (0·95)	95·9 (85–99)	0·07	8·629	0·003
		0·25[a]	1	1·75 (2·2)	90·4 (75–97)			
		0·125[a]	2	1·5 (2·4)	91·8 (77–98)			
		0·0625[b]	0	6·8 (4·0)	24·5 (0–64)			
	Ivermectin	1[a]	3	0·25 (0·5)	98·6 (90–100)	0·11	11·324	<0·001
		0·5[b]	2	1·5 (2·4)	83·3 (48–96)			
		0·25[b]	1	1·25 (0·95)	86·1 (54–97)			
		0·125[c]	0	11·0 (3·3)	53·7 (23–72)			
	Levamisole	10[b]	4	0	100 (85–100)	1·05	17·826	<0·001
		5[b]	4	0	100 (85–100)			
		2·5[c]	3	0·25 (0·5)	98·9 (93–100)			
		1·25[d]	0	4·0 (3·2)	61·5 (16–83)			
	Albendazole	10[c]	0	11·25 (3·8)	52·6 (22–72)	9·8	0·456	0·499
		5[d]	0	9·8 (4·6)	6·3 (0–45)			
Combination chemotherapy	PF1022A 0·0625 mg/kg plus albendazole 10 mg/kg[d]		1	11·5 (14·7)	0 (0–38)		—	—
	PF1022A 0·0625 mg/kg plus ivermectin 0·125 mg/kg[d]		1	4·3 (3·3)	59·1 (12–82)		0·968	0·325
	PF1022A 0·0625 mg/kg plus levamisole 1·25 mg/kg[d]		1	3·5 (3·9)	66·3 (24–86)		2·215	0·137

[a] Versus control 1.
[b] Versus control 2.
[c] Versus control 3.
[d] Versus control 4.

in vitro, with treated adult *H. bakeri* comparable to control worms. The inability of ivermectin to affect adult *H. bakeri in vitro* was also confirmed in our egg release studies. However, interestingly, ivermectin achieved almost complete adult worm burden reductions *in vivo*, a finding, which is in line with previous publications (Wahid *et al*. 1989; Njoroge *et al*. 1997). High worm burden reductions against adult *H. bakeri* in mice were also observed with PF1022A at doses as low as 0·125 mg/kg. A previous study has shown that the semi-synthetic derivative of PF1022A, emodepside was not only active against the adult stages of *H. bakeri* but achieved complete worm burden reduction, when administered as early as 2 days post-infection (Harder and von Samson-Himmelstjerna, 2001). Cure of *H. bakeri*-infected mice was also observed with levamisole at 5 and 10 mg/kg confirming previous results (Chimwani and Britt, 1986). The lowest activity against *H. bakeri in vivo* was seen with albendazole.

The egg hatch test is a common method to detect benzimidazole and levamisole resistance (von Samson-Himmelstjerna *et al*. 2009). The incubation of *H. bakeri* eggs with our test drugs revealed an ovicidal effect of PF1022A and levamisole. Albendazole inhibited egg development at all tested concentrations.

To our knowledge we have, for the first time, tested the effect of PF1022A drug combinations against *H. bakeri in vitro* and *in vivo*. None of the drug combinations tested showed additive or synergistic but rather antagonistic effects on L3 larvae movement. These findings were also confirmed in our *in vivo* studies. None of the combinations tested in our study resulted in significantly higher worm burden reductions when compared to monotherapy. On the other hand, additive effects were observed in *H. bakeri*-infected mice in a previous study when emodepside was combined with piperazine (Nicolay *et al*. 2000). Why PF1022A showed decreased

activities, when combined with albendazole, levamisole or ivermectin cannot be explained at the moment, hence further studies should be launched.

In conclusion, our work confirmed that *H. bakeri* is an excellent model to study the anthelmintic activity of test drugs. We have demonstrated that L1/L2, L3 larvae as well as adult *H. bakeri* are suitable stages for *in vitro* testing. Our study provides a rationale to further study PF1022A as a potential novel anthelmintic drug for the treatment of STH. We documented that PF1022A has significant larvicidal and nematocidal activity *in vitro* and the drug revealed the highest activities *in vivo* when compared to the reference drugs albendazole, levamisole and ivermectin. In the light of our promising findings with PF1022A further investigations should be launched possibly in a next step against the target nematodes the hookworms, *Trichuris* spp., *Strongyloides* spp. and *Ascaris* spp. in permissive hosts.

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