Molecular & Biochemical Parasitology 180 (2011) 86–98

Contents lists available at SciVerse ScienceDirect

Molecular & Biochemical Parasitology

Identification of phage display peptides with affinity for the tegument of *Schistosoma japonicum* schistosomula☆

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ARTICLE INFO

Article history:
Received 26 February 2011
Received in revised form 31 August 2011
Accepted 5 September 2011
Available online 12 September 2011

Keywords:
Schistosoma japonicum
Phage display technology
M13 phage peptide ZL4 (MppZL4)
Ectogenic polypeptide
Schistosomicidal effect

ABSTRACT

Peptides, bound to the tegument of live *Schistosoma japonicum* schistosomula, were differentially screened by phage display *in vitro* using three rounds of reverse absorption and bio-panning. Three M13 phage peptides were isolated and identified by determination of their recovery rate, immunohistochemical localization, immunoblot analysis, and their anti-schistosomal effects *in vivo* and *in vitro*. Of the three, M13 phage peptide ZL4 (MppZL4, YSGLQDSSLRLR, 1.4 kDa, p*I* 8.8) bound to the tegument of mechanically transformed schistosomula and to other developmental stages of *S. japonicum* from the mammalian host. By contrast, MppZL4 did not bind to the surface of cercariae. To further examine its binding properties, MppZL4 was conjugated to Rhodamine B (RhB-YSGLQDSSLRLR, RhB-ZL4) and a peptide control (RhB-AIPYFSGILQWR, RhB-12P) was similarly synthesized. The binding capacities of RhB-ZL4 to the surface membrane of *S. japonicum* schistosomula *in vitro* and of *S. japonicum* adult worms *in vivo* were examined and revealed specificity for binding. When examined for anti-parasite activity, both MppZL4 and RhB-ZL4 exhibited a potent schistosomicidal effect *in vitro*. Further MppZL4 also affected the growth and development of schistosomula *in vivo*. These findings extend previous studies showing that phage display techniques can recover polypeptides that bind specifically to living schistosomes and, moreover, that these bound peptides have the potential to inhibit key physiological processes in these parasites. Our findings suggest further that ectogenic polypeptides, which can bind to the tegument of *S. japonicum*, might be adapted as vectors to deliver experimental probes and/or pharmacologically relevant compounds to the schistosome tegument, including drugs and immunological mediators.

1. Introduction

Schistosomiasis, a tropical infectious disease caused by the blood flukes *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*, infects 200 million people with 800 million more are at risk [1]. Although methods for control and treatment of schistosomiasis are available, including mass chemotherapy of target populations with praziquantel, new interventions are needed in light of the rapid reinfection after drug treatment and because of the potential of drug resistance [2]. Schistosomes are obligate parasites and genome sequence analysis has revealed they have evolved to rely on host-derived components for many aspects of their biology and physiology [3]. The adult stages of schistosomes reside in the blood stream of their definitive host, where successful growth, development, reproduction and survival depend on the provision of nutrients and host shelter. Blood flukes obtain nutrients from their hosts across two parasite surfaces, the tegument and the gastrodermis of the gut. In addition, host antigens and other macromolecules are identifiable on the schistosome surface [4], a phenomenon hypothesized to enhance evasion of host immunological responses [5]. The schistosome tegument is a living syncytium, bounded by a complex multilaminate surface. On its internal surface, the tegument is bounded by a basal membrane whereas the apical plasma membrane covers the extremity of the worm. A matrix-like

☆ *Financial support*: This study was supported financially by the Natural Science Foundation of China (Grant No. NSFC:30570952), the Hunan Provincial Science Foundation (10JJ3010), the Foundation of Hunan Provincial Health (B2009051), the Key Projects Fund of Central South University (2010QZZD027) and the Key Projects Fund of Changsha City (K0802105-31).
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doi:10.1016/j.molbiopara.2011.09.001

material is located between the membranes [6]. The basal membrane and the outer membrane are constituted of a double-unit membrane. Tegumental cell bodies extend to the muscular layer under the body wall. The surface of the tegument is convoluted in form, and includes tegumental ridges and pits [7].

Schistosome growth, development and reproduction also requires absorption from the host of cholesterol, phospholipids, thyroxin, sex hormones and insulin, and likely many other host components which schistosomes cannot synthesize de novo [8–10]. Many of these nutrients and also much of the communication at the host–parasite interface including host ligand–parasite receptor interactions takes place at the schistosome tegumental surface [8,11–16]. It is thought that the schistosome parasite not only binds host ligands to its own schistosome receptors to avoid immune attack [17–19] but that it relies also on host immune mediators to regulate its growth and development [20]. In addition, the schistosome tegument is constantly being released and this shedding of antigens stimulates the host to produce an immune response.

The schistosomal tegument is the primary interface with host blood in the context of nutrient absorption, immune evasion, signal transduction, secretion of metabolites and osmotic pressure homeostasis [21]. Accordingly, numerous investigations have targeted the tegument for development of anti-schistosomal interventions including vaccines [22] and diagnostics. At the same time, it is apparent that its structure and function has similarities with that of mammalian cell membranes. Thus we speculated that phage display technology could be used to bio-pan out specific peptide molecules which may be absorbed by the parasite surface being activated, based on the principle of receptor–ligand binding or charge difference binding. The properties of a peptide that binds specifically to the schistosome tegument could be to mimic ligands, epitopes or small molecules important in the host–schistosome relationship. These binding components might represent agonists or antagonists of parasite growth and development. Hypothetically, a promoting molecule might be represented by an essential ligand epitope whereas an antagonist might be a small molecule that could block an essential ion channel. However, regardless of its role, as long as there is specific binding, the peptide is worthy of investigation as a drug target or for characterizing biochemical, biophysical and immunological aspects of the schistosome surface.

Here we explored exogenous peptides absorbed by the schistosome tegument under physiological conditions, and their properties. Schistosomula transformed mechanically from cercariae of *S. japonicum* were studied using an affinity bio-panning adaptation of the phage display technique. Screening yielded a peptide termed ZL4 that bound to the tegument of mammalian stages of *S. japonicum* but not to the surface of cercariae. Intriguingly, ZL4 antagonized growth and development of *S. japonicum* in mice and was lethal to schistosomula *in vitro*.

2. Materials and methods

2.1. Schistosomes, snails, rodents, reagents

All animal care and procedures were conducted according to the guidelines for animal use in toxicology (Society of Toxicology USP, 1989). A mainland strain of *S. japonicum*, maintained in *Oncomelania hupensis* snails, was provided by the Hunan Institute of Schistosomiasis Control, Yueyang, Hunan Province, PR China. Female Kunming mice, 6–8 weeks old, were purchased from Central Southern University Animal Unit (Changsha, Hunan Province, PR China). A Ph.D.-12™ Phage Displayed Peptide Library Kit [1.9×10^{13} plaque forming units per ml (pfu/ml), contains a complexity of 2.7×10^9 individual M13 phage clones] (Fig. S1(B)), M13KE Phage (Fig. S1(A)) as negative control and *Escherichia coli* 2738 (ER2738) were purchased from New England BioLabs (Beverly, MA, USA). RPMI-1640 medium was purchased from Invitrogen (Carlsbad, CA, USA). Mouse monoclonal antibody against M13 (M13 McAb) was purchased from Abcam (Cambridge, United Kingdom). Horseradish peroxidase-conjugated monoclonal antibody against M13 (HRP-M13 McAb) was purchased from GE Healthcare (Bio-Sciences AB, USA). HRP-IgG McAb was purchased from Beyotime (ShangHai, China). Phage DNA sequencing was performed by Invitrogen (Shanghai, China).

2.2. Differential screening with a phage peptide library

2.2.1. Obtain the cercariae and schistosomula

Cercariae were shed from snails and collected under axenic conditions, centrifuged ($1000 \times g \times 10$ min), washed 3 times in sterile Phosphate-buffered Saline (PBS) and 3 times with RPMI 1640 (Invitrogen, USA). Cercariae were divided into two groups – one group was directly used for construction of a reverse adsorption peptide library, the other for bio-panning (forward screening) the peptide library after mechanical transformation to schistosomula [23,24]. A 5 ml suspension of cercariae was dispensed onto a small flat plate, and the suspension was aspirated 20 times using a 10 ml-injection syringe with a 23G gauge needle. Microscopic examination indicated that more than 85% of the cercariae had transformed into schistosomula. The worms and their tails were separated by gently shaking the small plate, and the tails were removed using a Pasteur pipette under a stereomicroscope.

2.2.2. Cercariae pre-reverse adsorption peptide library

Three μl of original phage containing 5.70×10^{10} plaque forming units (pfu) and 500 ± 20 cercariae in 1 ml ddH_2O were dispensed into 15 ml centrifuge tubes at 37 °C and the tubes gently agitated for 150 min. The supernatant (circa 1 ml), containing unbound phage was collected by centrifugation ($1000 \times g \times 10$ min) and then 170 μl PEG/NaCl, containing 20% (w/v) PEG-8000 and 2.5 M NaCl, was added and the tubes were kept at 4 °C overnight to precipitate the phages. The precipitated pellet was collected by centrifugation ($12{,}000 \times g \times 15$ min; 4 °C) and taken up in 100 μl Tris-buffered saline (TBS). To quantify the number of phage clones in the supernatant, 1 μl was titrated by Real-time Polymerase Chain Reaction (Real time PCR), and then the "Yield rate" was calculated using the formula: Yield rate = Phage in supernatant after reverse adsorption/total phage added to the cercariae × 100%.

2.2.3. Real time PCR Assay

Primer 3.0 (http://frodo.wi.mit.edu/) was used to construct primers based on the sequence of the M13 phage. The primer sequences were chosen to yield an application length of 122 bp, which was confirmed in a standard PCR in which the application product was visualized on an agarose gel. Primer A was 5′-TTCGCAATTCCTTTAGTGGT-3′ and primer B 3′-TGGGATTTTGCTAAACAACTTTC-5′. Phage templates were heated to 100 °C for 5 min then cooled on the ice before use [25,26]. As standard templates, 1 μl of the original phages were 10-fold serially diluted from 10^{10} to 10^1 and PCR amplification undertaken so as to provide a standard curve for the M13 phage.

The Real-time PCR mixture included 1 μl phage template, 0.6 μl primer A, 0.6 μl primer B and 2 × SYBR Green PCR Master mix (ABI, USA), with ddH_2O added to a final volume of 20 μl. PCR was performed on an ABI Prism 7500. The PCR program consisted of an initial melting step of 30 s at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C, and then the fluorescence signals were collected during the annealing step of each cycle. After amplification, a melting curve was produced by cooling the products rapidly to 60 °C, holding them for 45 s, and then heating them

at 0.2 °C/s to 95 °C. Each point of the curve and each sample were amplified in triplicate. As a blank control, ddH_2O, without any template, was included for each run, while the cDNA of human GAPDH (Revert Aid H Minus First Strand cDNA Synthesis Kit, ProMab, USA) – using 5′-AGAAGGCTGGGGCTCATTTG-3′ as forward primer and 5′-AGGGGCCATCCACAGTCTTC-3′ as reverse primer, giving a product of 258 bp, acted as positive control. Data were analyzed using curve expert 1.3 software with the standard curve obtained for the M13 phage.

2.2.4. Phage amplification

The phages from the supernatant were amplified to produce sufficient numbers for the next experiment. The supernatant added to 20 ml ER2738 culture and incubated at 37 °C with vigorous shaking for 4.5 h. Phage amplification, purification and concentration were carried out using instructions from the manual of the display peptide library.

2.2.5. Schistosomula bio-panning peptide library

Transformed schistosomula (500 ± 20) were washed 3 times in TBS, blocked with blocking buffer (TBS, 0.5% Tween, 3% w/v BSA) at 37 °C for 1 h, then placed in 15 ml centrifuge tubes with $\sim 10^{10}$ pfu amplification phage, and the mixture agitated for 150 min. Supernatants, including unbound phages, were removed by centrifugation and the worms were washed 10× with TBST containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.1% (v/v) Tween 20. Subsequently, 770 μl glycine–HCl–BSA (0.2 M Glycine–HCl, 1 mg/ml BSA, pH 2.2) was added to the centrifuge tubes with the schistosomula and the mixture agitated gently for 5 min to elute bound phages from the schistosomula. The eluted supernatants were collected by centrifugation (1000 × *g* × 10 min), 230 μl Tris–HCl (pH 9.1) was added in order to neutralize the mixture, and then 170 μl PEG/NaCl was added to the tubes to precipitate phages. The precipitated pellet was collected by centrifugation and taken up in 100 μl TBS. In order to determine the number of phages present, 1 μl of the eluted phage were used for phage titer assays with Real time PCR.

Residual eluted phages were amplified as above for the next round of reverse absorption and bio-panning.

2.2.6. Second and third rounds of differential screening

The procedures of reverse adsorption, amplification, bio-panning and amplification of phages for the second and third rounds of differential screening were essentially the same as the first round. The main differences were that the phages used were selected from the previous round following their elution from schistosomula and amplification, and that the Tween-20 concentration in TBS was raised to 0.5% (v/v) for the binding and washing steps.

The supernatant-phages and the eluted phages from the first and second rounds were amplified and used for the subsequent round of screening, respectively. Eluted phages from the third round were not amplified. Fifteen individual phage clones were randomly picked from the third round of differential screening on LB/IPTG/X-gal plates [1L LB medium including 15 g agar, 0.05 g IPTG (isopropyl-β-D-thiogalactoside) and 0.04 g X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)], and were sequenced after plaque amplification.

2.3. DNA extraction, sequencing and analysis

Phage single strand DNA was isolated using sodium iodide (NaI) according to the kit's instructions (New England BioLabs) after the 15 phage clones had been randomly selected from LB/IPTG/X-gal plates and amplified in LB medium. Phage clones were identified by sequencing. The primer used to obtain the sequence of the peptide insert was 5′-CCCTCATAGTTAGCGTAACG-3′. For each DNA sequence, the amino acid composition, hydrophilicity, isoelectric point (p*I*) and molecular weight (MW) were predicted with the assistance of bioinformatics tools (http://br.expasy.org/tools/pi.tool.html).

2.4. Recovery rate of positive phage clones

Three positive phage clones, named M13 phage peptide ZL1, M13 phage peptide ZL4 and M13 phage peptide ZL6 (MppZL1, MppZL4 and MppZL6), which were identified as a result of differential screening, and a negative control phage, termed M13KE phage, were used to test the recovery rate in order to determine their binding ability to schistosomula. Viable schistosomula (500 ± 20), which had been centrifuged, washed with TBS, and blocked with blocking buffer (TBS, 0.5% Tween, 3% w/v BSA) at 37 °C for 1 h, were incubated with the positive phages in 15 ml-centrifuge tubes. Thereafter, $\sim 10^{10}$ pfu phage clones were added and the mixture incubated with shaking for 150 min at 37 °C. The schistosomula were washed 10 times with TBST-0.5% by centrifugation at 1000 × *g* for 10 min to remove unbound peptides. Phages bound to the parasites were eluted with 770 μl glycine–HCl–BSA, neutralized with 230 μl Tris–HCl, and precipitated with PEG/NaCl. 1 μl of the eluted phage were used for phage titer assays using Real time PCR. The recovery rate was calculated using the formula: recovery rate = $pfu_e/pfu_t \times 100\%$, with pfu being phage eluted from the schistosomula, and pfu_t being the total phage added to the schistosomula.

2.5. Specific binding of positive phage clones to the tegument of schistosomula shown by western blot

2.5.1. The binding capacity of MppZL0, MppZL1, pp ZL4 and MppZL6 to schistosomula

At 18 days post-infection with 100–120 cercariae, a total of 180 mice were assigned to six groups, 30 mice each group, and injected, respectively, with four clones [MppZL0 (without the inserted fragment), MppZL1, MppZL4 and MppZL6] as 4 test groups ($\sim 10^{12}$ pfu phages each mouse); the M13KE phage ($\sim 10^{12}$ pfu) as negative control; and one group with 100 μl TBS as an injection control. Fifteen min after injection, all mice were euthanized in order to collect worms. 0.5 g of worms from each group were recovered from the mice by perfusion of the mesenteric blood vessels, and washed 10 times with TBS-0.5% (v/v) Tween. Schistosome tegumental proteins were extracted with 100 μl RIPA Lysis buffer (Beyotime, China) containing 1 mM of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) for 30 min at 4 °C. The mixture containing worms and RIPA was centrifuged at 15,000 × *g* for 5 min, and the supernatant containing the tegumental proteins of *S. japonicum* were collected and kept at −80 °C until used. At the same time, the total protein of M13 phage (Fig. S1(B)), as a positive control, was prepared with $\sim 10^{12}$ pfu M13 phage precipitated with PEG/NaCl. The total proteins of M13 phage were extracted with 80 μl RIPA Lysis buffer containing 1 mM PMSF for 30 min at 4 °C, and the mixture containing M13 phage and RIPA were centrifuged (15,000 × *g* × 5 min) to collect the supernatant and the total M13 phage protein. Protein concentrations were determined using the BCA system (Pierce, Rockford, IL, USA).

Immunoblot analysis was undertaken using a Minigel system (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (about 50 μg) were separated on 10% (w/v) SDS–PAGE at 20 mA constant current electrophoresis at room temperature and electrotransferred to nitrocellulose membrane (NC, Pall Corporation) at 120 volts for 4 h at 4 °C. To block non-specific binding, the membrane was incubated in TBS with 0.1% TBST containing 5% (v/v) nonfat milk for 2 h. After washing in TBST, M13 McAb was applied

(1:5000, in TBST with 2% BSA, w/v) and incubated overnight at 4 °C. Be washed 3 times, the samples were added HRP-IgG McAb (1:1000) and incubated 120 min at 37 °C. Enhanced chemiluminescence (ECL, Santa Cruz) was used to visualize the reactions. Each sample was also probed with a rabbit anti-β-tubulin antibody (ABCam, USA) as a loading control for the strong homology (Identities = 96%, Positives = 99%) between β-tubulin of *S. japonicum* (AY220457) [27] and mice (NM_146116) [28]. The bands were analyzed by Volum Contour of Quantity One Software (Bio-Rad Laboratories, Hercules, CA, USA).

2.5.2. Fractions of schistosomula tegument protein binding to MppZL4

Ten mice were each infected with 200 ± 5 *S. japonicum* cercariae. At 18 days post-infection, the animals were euthanized in order to collect schistosome worms. The worms were recovered from the mice by perfusion of the mesenteric blood vessels, and washed 10 times with TBS. The tegumental proteins of the schistosome worms were extracted with RIPA Lysis buffer. Protein concentrations were determined using the BCA system. Equal amounts of protein (50 μg) were separated on 10% (w/v) SDS–PAGE and electrotransferred to NC membrane. The membrane was blocked with 4% (v/v) fetal bovine serum. The MppZL4 ($\sim 10^{12}$ pfu/ml) was applied and incubated overnight at 4 °C. HRP-M13 McAb was then applied (1:2500 in TBS with 2% BSA, w/v) and the membrane incubated for 120 min at 37 °C after the membranes had been washed 3 times in TBS. Enhanced chemiluminescence was used to visualize reactivity. At the same time, the M13KE phage was used instead of MppZL4 as negative control.

2.6. Positive phage localization analysis by immunohistochemistry

Of the three positive phage clones, MppZL4 was selected for immunolocalization to discrete developmental stages-cercariae, schistosomula transformed from cercariae, lung schistosomula, liver schistosomula and adult worms with M13 McAb as this provided the highest recovery rate among the three clones. M13KE phage was used as negative control in parallel immunolocalization experiments.

2.6.1. Localization to cercariae and schistosomula in vitro

Cercariae and schistosomula were prepared as above; 500 ± 20 viable schistosomula and 500 ± 20 active cercariae were mixed in 15 ml-centrifuge tubes with $\sim 10^{10}$ pfu MppZL4 or $\sim 10^{10}$ pfu M13KE phage (as negative control), and incubated with gentle shaking at 37 °C for 150 min. The parasites were fixed with 4% (w/v) paraformaldehyde (PFA) for 10 min, after which they were washed 10 times with TBS-0.5% Tween. The worms were agitated gently, placed carefully on a glass slide (two slides/reaction sample) and dried briefly with a fan. 100 μl M13 McAb diluted 1:200 in TBST was added and incubated overnight at 4 °C. After washing 3 times, 100 μl of 1:50 diluted horseradish peroxidase-labeled goat anti-mouse IgG (HRP-IgG McAb, Beyotime, China) were added and incubated for 30 min at 37 °C. The worms were washed 3 times with TBS before color was visualized after 10–30 min at room temperature using freshly prepared 3,3′-diaminobenzidine (DAB, Sigma Chemicals, St. Louis, MO, USA) solution. The color reaction was terminated with distilled water. Finally, one slide of each reaction sample was stained with hematoxylin [29,30]. Images were obtained using digital system micro-photography (Nikon, Japan). The number of positive reactions (deep brown color appearing on the parasite surface membrane) in 100 worms was recorded for each reaction sample.

2.6.2. Localization to worm sections of developmental stages from mice

Twenty-seven Kunming mice were infected with *S. japonicum* cercariae (200 ± 10/mouse). The mice at day 3 post-infection were randomly divided into three groups (nine mice each group) and were injected respectively with $\sim 10^{12}$ pfu MppZL4 (approximately 0.3 mg as positive group), $\sim 10^{12}$ pfu M13KE phage (as negative control group) and 100 μl TBS (as blank control group) per mouse through the caudal vein [31]. Fifteen minutes post-injection, the lungs of mice were collected to obtain lung schistosomula. Livers of mice were collected for liver schistosomula at 10 days post-infection while adult worms were collected by perfusion from 24-day infected mice. The tissues and worms were fixed, embedded in paraffin and serial sectioned. After deparaffinization, endogenous peroxidase was inhibited by treatment with 10% H_2O_2-methanol in PBS at room temperature for 30 min. Sections were washed twice by immersion in PBS for 10 min. The sections were incubated for 1 h at 37 °C with M13 McAb (diluted 1:200) and washed 3 times with PBS. Be washed 3 times, the samples were added HRP-IgG McAb diluted 1:50 and incubated 30 min at 37 °C. Peroxidase activity was visualized using DAB as the substrate; the reaction was terminated after 15 min by rinsing with water, and the sample was stained with hematoxylin. Finally, the slides were mounted in aqueous-based mounting medium and observed under light microscopy. A positive result was based on deep brown staining on the parasite surface. Positivity rates were counted for each reaction sample. Portions of the serial sections were stained with hematoxylin–eosin (HE) for structural observations.

2.7. Positive peptide RhB-ZL4 synthesis and localization analysis

The Rhodamine B-conjugated peptide ZL4 (RhB-YSGLQDSSLRLR, RhB-ZL4) and Rhodamine B-conjugated 12 random peptide (RhB-AIPYFSGILQWR, RhB-12P) as control were synthesized and purified by ChinaPeptides Co., Ltd. (Shanghai, China). The purity of RhB-ZL4 was 98%.

2.7.1. Binding ability of RhB-ZL4 to schistosomula in vitro

Schistosomula were prepared as above and transferred (100 ± 5 schistosomula and cercariae per well in 400 μl medium) to wells of 48-well cell culture plates (Corning Glass, Corning, NY). The 48 wells were assigned to three groups: RhB-ZL4 (20 μg) as the experimental group, RhB-12P (20 μg) as the negative control group and TBS (20 μl) as blank control group [32,33]. The schistosomula were incubated with gentle shaking at 37 °C for 150 min. The parasites were collected and then washed 10 times with TBST by centrifugation (1000 × *g* × 10 min). Whole-mount parasites were examined using a fluorescence microscope (Nikon ECLIPSE, TE 2000-S, Japan) and photos taken [34–36]. The number of positive reactions in 100 worms was recorded for each reaction sample.

2.7.2. Effect of RhP-ZL4 binding to S. japonicum in vivo

The RhP-ZL4 was diluted to 1 mg/ml with saline. Thirty mice infected with 200 ± 10 cercariae were assigned randomly to three groups, 10 mice per group. At 24 days post-infection, the 300 μg RhB-ZL4 (I group, as experimental group), 300 μg RhB-12P (II group, as negative control group) and 300 μl TBS, (III group, as blank control group) were injected into the tail vein of the infected mice, respectively [37]. The mice were euthanized 15 min post-injected, and worms were recovered from the portal circulation by perfusion with saline introduced into the heart after the hepatic portal vein had been severed. Whole-mount parasites were examined using a fluorescence microscope and photos taken after being washed 10 times with TBST [36]. Furthermore, some parasites were agar-embedded, frozen then sliced, and observed under a fluorescence

microscope [32,38]. The number of positive reactions in 100 worms was recorded for each group.

2.8. Lethal effect of MppZL4 and RhB-ZL4 in vitro

S. japonicum cercariae, collected under axenic conditions and washed with sterile TBS, were mechanically transformed to schistosomula. The schistosomula were washed three times with RPMI 1640 by centrifugation (1000 × *g* × 5 min). After the density of the schistosomula was adjusted to ~1000/ml using RPMI 1640 medium containing penicillin (100 UI/ml) and streptomycin (100 μg/ml), schistosomula (100 ± 5 worms/well) were transferred to the wells of 48-well cell culture plates. The 48 wells (100 ± 5 schistosomula per well in 200 μl medium) were assigned to five groups, 3 wells per group: TBS as blank control group; M13KE (~10^{10} pfu, about 20 μg) and RhB-12P (20 μg) as the negative control groups and MppZL4 (~10^{10} pfu, about 20 μg) and RhB-ZL4 (20 μg) as the experimental groups, separately. The schistosomula were maintained at 37 °C for 72 h under 5% CO_2 [39]; mortality was evaluated every 24 h with the aid of an inverted microscope and methylene blue [MB, 0.5% (w/v)] staining; killed worms stained blue whereas viable worms remained mobile, translucent and generally free of blue staining [40]. The experiment was repeated under identical conditions to confirm the results.

2.9. Effect of MppZL4 on S. japonicum development in vivo

Forty mice infected with 30 ± 1 cercariae were assigned randomly to four groups. The phage peptide ZL4, positive group (10^{12} pfu, about 0.3 mg), M13KE negative control (10^{12} pfu, about 0.3 mg) and TBS (100 μl), blank control, were injected into the tail vein of the infected mice on days 1–7 post-infection, whereas mice in the positive control group were treated with praziquantel (PZQ) at 300 mg/kg administered by stomach intubation 36 days after infection. The mice were euthanized at 70 days post infection, and worms were recovered from the portal circulation by perfusion with saline introduced into the heart after the hepatic portal vein had been severed. Worms were counted and each pair of male and female worms weighed using a 1/10,000 electronic balance (Shimadzu, Japan). The livers were removed and processed for egg counts [41] which were expressed as liver eggs per gram (LEPG). The worm reduction rate, worm weight reduction rate and the LEPG reduction rate were calculated according to the following formula: [(the average number of negative control group – the average number of test group)/the average number of negative control group] × 100%. The experiment was repeated three times.

2.10. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) or by χ^2-tests using the R × C table. *P* values of ≤0.01 were considered to be statistically significant.

3. Results

3.1. Differential screening of phages binding to schistosomula

Phage peptides that bound to schistosomula transformed from cercariae, but did not bind to cercariae, were isolated using three cycles of reverse absorption and bio-panning *in vitro*. In each cycle, whereas the unbound phages were cleared by washing with TBST, bound phages were recovered from schistosomula and amplified in *E. coli* for subsequent enrichment cycles. The phage titer was titrated using Real time PCR (Fig. S2). Both phage titer and recovery efficiency were improved substantially following each round of reverse absorption and bio-panning (Table 1). After three rounds of *in vitro* differential screening, the rate of phages recovered from schistosomula increased 941-fold (from 0.000034% to 0.032%) compared with the recovery rate in the first round. On the other hand, there was a decrease in the number of phages binding to the cercariae as the yield rate of the supernatant was increased substantially (from 0.0017% to 73.51%). These results indicated an obvious enrichment of phages specifically binding to the schistosomula.

3.2. Novel peptides encoded by phage bound to schistosomula

After three cycles of reverse absorption and bio-panning, 15 peptide clones were randomly picked from all positive peptide clones (Fig. S3(A)). DNAs were extracted from these clones and their sequence determined (Fig. S3(B–D)). Bioinformatics analysis showed different biological characteristics of the peptides encoded by the 15 positive phages (Table 2). We noted MppZL4, with the insert amino acid sequence NH_2-YSGLQDSSLRLR, was represented four times in the 15 positive clones.

3.3. The MppZL4 bound tightly to schistosomula

To confirm the binding ability of positive phages, the MppZL1, MppZL4, MppZL6 and negative control M13KE phage were tested for recovery rate. Data were analyzed for significance using the χ^2-test and R × C table, assisted by the SPSS version 11.5 software package. Significantly higher binding was observed by MppZL4 to schistosomula than by MppZL6 or MppZL1 ($P < 0.01$) or control M13KE phage ($P < 0.00$) (Table 3).

Table 1
Phage enrichment after each round of reverse absorption and bio-panning.

Round	Phage added (×10^7 pfu)	Phage in supernatant after cercariae absorption		Phage eluted from schistosomula (bio-panning)	
		Pfu (×10^7)	Yield rate[a] (%)	Pfu (×10^7)	Recovery rate[b] (%)
1st reverse absorption	5700.00[c]	0.098 ± 0.000052	0.0017		
1st bio-panning	2610.04 ± 96.12[d]			0.00090 ± 0.0000084	0.000034
2nd reverse absorption	4189.12 ± 47.24[e]	1.50 ± 0.0054	0.0357		
2nd bio-panning	3703.70 ± 14.02[d]			0.0067 ± 0.00064	0.0018
3rd reverse absorption	5427.25 ± 66.98[e]	3991.70 ± 7.84	73.51		
3rd bio-panning	3093.66 ± 26.19[d]			0.985 ± 0.0077	0.032

[a] Yield rate = Phages in supernatant after reverse adsorption/total phages added to the cercariae × 100%. This can also be interpreted as the percentage rate of the free phages not adsorbed by cercariae from reverse adsorption of the total number of phages added.
[b] Recovery rate = phages eluted from the schistosomula/total phages added to the schistosomula × 100%.
[c] Phages were from the original peptide library.
[d] Phages from the supernatant after cercariae reverse absorption and amplified on the phage volume.
[e] Phages from last round of bio-panning and amplified on the phage volume.

Table 2
Biological characteristics of the peptide molecules from 15 positive phage clones.

No.	The insert amino acid sequence (N → C)	Acidic AAR[a]	Basic AAR	Hydrophilic polar AAR	Hydrophobicity nonpolar AAR	Name	pI	MW (Da)
zl4-1, zl4-4, zl5-2, zl5-3, zl5-4, zl7-1	DRYSEIRTSSTL	D, E	R, R	Y, S, S, S, T, T	I, L	ZL6	6.07	1427.53
zl4-2, zl5-1, zl5-5, zl7-2	YSGLQDSSLRLR	D	R, R	Y, Q, S, S, S	G, L, L, L	ZL4	8.75	1394.55
zl4-3	DYPSANKWPRYV	D	K, R	Y, Y, S, N, W	P, P, A, V	ZL1	8.50	1495.66
zl5-6, zl6-4	No inserted fragment					ZL0[b]		
zl6-1, zl6-2	DAN degraded							

[a] AAR (amino acid residue).
[b] Phage ZL0 has no inserted fragment.

3.4. Binding of phage to the tegument of S. japonicum schistosomula

3.4.1. The binding capacity of phage clones ZL0, ZL1, ZL4 and ZL6 to schistosomula

Immunoblot analysis was undertaken to investigate potential differences in the capacity of MppZL0, MppZL1, MppZL4 and MppZL6 to bind to schistosomula. A pronounced band of ~45 kDa was evident in the protein fraction of schistosomula after reactivity with MppZL4 and the total protein from M13 phage (Fig. 1A, lanes 5 and 7); this molecular mass (~45 kDa) is consistent with the mass of the gp3 protein of M13KE phage [42]. The relative density (INT) of lane 7 was 321.7 and that of lane 5 was 131.5 obtained through Quantity One software (Fig. S4). Furthermore, *S. japonicum* β-tubulin (55 kDa) was evident in lane 5, as lanes 1, 2, 3, 4, 6 containing schistosomula proteins, showed the presence of β-tubulin but M13 phage did not. As blank control, the band was not apparent in the tegumental protein fraction of schistosomula without reactivity with any phage (Fig. 1A, lane 1), nor the tegumental protein of schistosomula reacted, respectively, with M13KE phages, MppZL0, MppZL1 and MppZL6 (Fig. 1A, lanes 2, 3, 4 and 6). The 55 kDa β-tubulin loading control was also evident in the immunoblot. The results indicate that peptides of the MppZL4 exhibited strong affinity for the surface membrane or tegument of schistosomula.

3.4.2. Schistosomula tegument proteins binding to MppZL4

Immunoblot analysis was undertaken to determine whether schistosomula tegument proteins were bound by MppZL4. In duplicate experiments, five bands with MWs ranging from 17 kDa to 28 kDa were observed after the schistosomula tegumental proteins were reacted with MppZL4 (Fig. 1B, lane 2). No bands were evident after the schistosomula tegumental proteins were incubated with M13KE phage as blank control (Fig. 1B lane 1). The results indicated that several small proteins in the schistosomula tegument of *S. japonicum* were recognized or bound by MppZL4.

3.5. The MppZL4 immunolocalized to the tegument of blood stage schistosomes

We investigated the affinity of MppZL4 for the surface of diverse developmental stages of *S. japonicum*, including cercariae and blood stage forms from experimentally infected mice. Strong reactivity was evident on worm surface membranes of intact schistosomula transformed from cercariae (Fig. 2A1 and A2); 92% of 100 schistosomula were positive. By contrast, no or, at most, minimal reactivity of MppZL4 was observed on intact cercariae (Fig. 2B1 and B2); 7% or less of 100 cercariae showed a positive signal. No binding (100% negativity) was observed of control M13KE phages to intact cercariae or schistosomula (Fig. 2C1 and C2).

Figs. 3 and 4 present the immunochemical results of MppZL4 and M13KE phage reactivity with lung schistosomula in the lung tissue and liver schistosomula in the liver tissue from infected mice. Strong positive reactivity was seen at the surface membrane of schistosomula with MppZL4 (Fig. 3A1 and Fig. 4A1 and B1; red arrow to area of positive reactivity). The positive rates of both were 87% in the13/15 sections of lung schistosomula and 90% in the 27/30 sections of liver schistosomula, respectively. No staining was seen with M13KE phage (Fig. 3B1 and Fig. 4C1 and D1); negative rates for both were 100% – in 12 sections from lung schistosomula and 25 from liver schistosomula. Immunostaining of adult blood flukes revealed positive reactions only with MppZL4 (Fig. 5A); 27 of 30 sections (90%) were positive for MppZL4. In summary, MppZL4 bound specifically to the teguments of schistosomula (lung and liver forms) and sexually mature adults of *S. japonicum*. By contrast, MppZL4 did not bind to schistosome cercariae.

3.6. Positive peptide RhB-ZL4 localization analysis by fluorescence

The results of the experiments described above suggested that the MppZL4 had specificity for *S. japonicum*. To investigate whether the free peptide maintained this binding affinity after removal from its phage protein framework, we made a synthetic peptide – ZL4-labeled with Rhodamine B. After the schistosomula and cercariae were incubated with RhB-ZL4, red fluorescence was evident on the outer membrane of the schistosomula, including the vicinity of the oral and ventral suckers (Fig. 6A1 and A2; a green arrow denotes the oral sucker and the white arrow point to the ventral sucker). In contrast, negative results were obtained with cercariae-binding-RhB-ZL4, and schistosomula-binding-RhB-12P. Occasionally, minor red fluorescence was seen on the oral and ventral suckers with schistosomula-binding-RhB-12P (Fig. 6B, shown by the green and the white arrows). RhB-ZL4 bound to the schistosomula on the surfaces of the tegument which suggested that peptide ZL4 may bind specifically to schistosomula, with a positivity rate of 78% (78/100 worms examine).

Whole-mount parasites were collected *in vivo* from infected mice, washed 10 times with TBST and examined with a fluorescence microscope. The adults of the I group, which were injected with RhP-ZL4, also showed positive fluorescent signals [83 of 100 worms (83%) were positive for RhP-ZL4] along the tegument of

Table 3
Differences in binding of three peptide phages to *S. japonicum* schistosomula.

Group	Total phage added to the schistosomula ($\times 10^7$ pfu)	Phage eluted from the schistosomula ($\times 10^7$ pfu)	Recovery rate (%)[a]
MppZL6	4318.27 ± 28.47	0.091 ± 0.00063	0.0021
MppZL4	4180.46 ± 35.29	1.91 ± 0.093	0.046
MppZL1	5514.49 ± 19.44	0.067 ± 0.00054	0.0012
M13KE phage	5143.84 ± 40.28	0.0000068 ± 0.00024	0.00000013

[a] Recovery rate = phage eluted from the schistosomula/total phage added to the schistosomula × 100%.

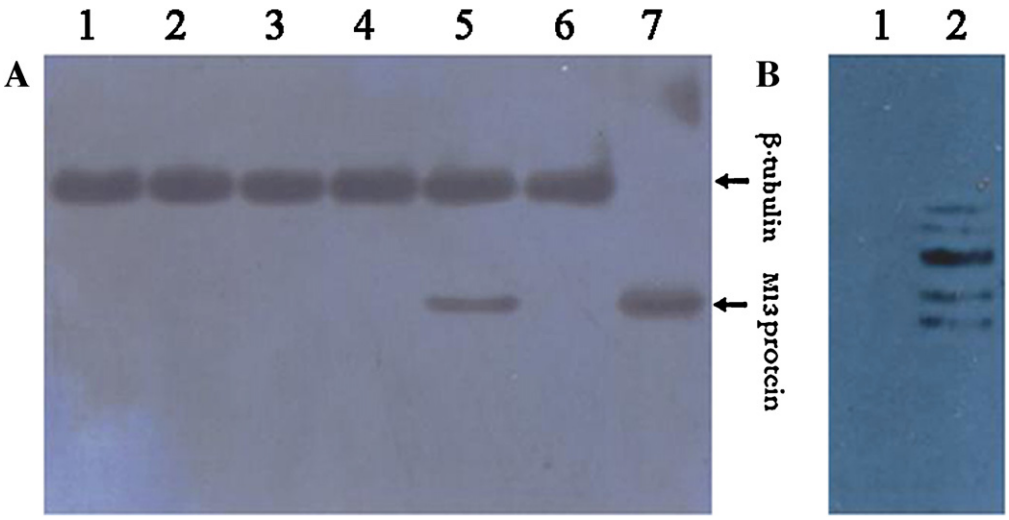

Fig. 1. Binding specificity of positive phage clones to the tegument of schistosomula by Western blotting. (A) The reactivity with phage clones that were injected through the tail vein of mice infected 18 days previously with *S. japonicum*. 15 min after injection, mice were euthanized and parasites recovered by portal perfusion. After washing, membrane proteins of the worms were extracted and used for immunoblotanalysis. Lane 1: tegument proteins from schistosomula (without addition of any phages). Lane 2: tegument proteins from schistosomula reacted with M13KE phage. Lane 3: tegument proteins from schistosomula reacted with MppZL0. Lane 4: tegument proteins from schistosomula reacted with MppZL1. Lane 5: tegument proteins from schistosomula reacted with MppZL4. Lane 6: tegument proteins from schistosomula reacted with MppZL6. Lane 7: total protein from M13 phage. A 45.0 kDa positive band is evident in lanes 5 and 7 while the 55.0 kDa β-tubulin protein as loading control is present in lanes 1–6. (B) Tegumental proteins extracted from schistosomula obtained from mice 18 days post-infection. The tegumental proteins were separated by SDS–PAGE and transferred to NC membrane to determine which fractions of the schistosomular tegument extract were bound by the MppZL4 using immunoblotting. The results of the immunoblot analysis showed that five bands of MW ranging from 17 kDa to 28 kDa were evident after the schistosomula tegument extract was reacted with MppZL4 (B, lane 2). No bands were observed when the tegument extract was reacted with M13KE phage (B lane 1) as negative control. An identical result was obtained when the immunoblot analysis was repeated.

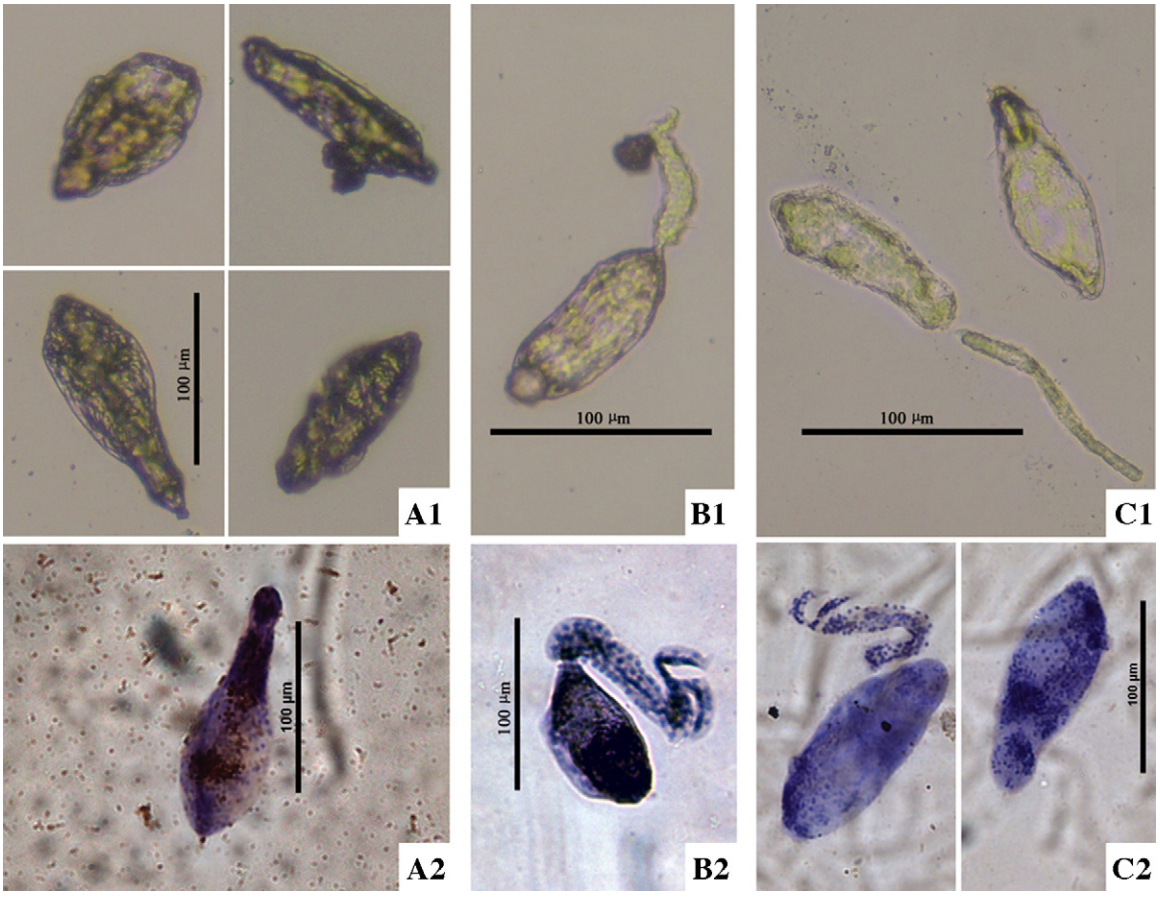

Fig. 2. Binding specificity of MppZL4 to cercariae and schistosomula *in vitro* analyzed by immunohistochemistry. Viable cercariae and mechanically transformed schistosomula were incubated with MppZL4 and M13KE phage, washed with TBS, fixed with 4% PFA and stained for histochemistry (HRP-anti-M13 mAb for recognition and DAB as the chromogenic substrate). Worm samples in panels A1, B1 and A2, B2 were probed with ppZ14; in panels C1 and C2 with M13KE phage. A2, B2 and C2 were from worms stained with hematoxylin after immunohistochemical staining. No brown-negative reaction was observed on cercariae (B1 and B2), whereas a dark brown positive reaction was evident in both A1 and A2. The deep red color in A2 resulted from immune staining with MppZL4, and then with hematoxylin.

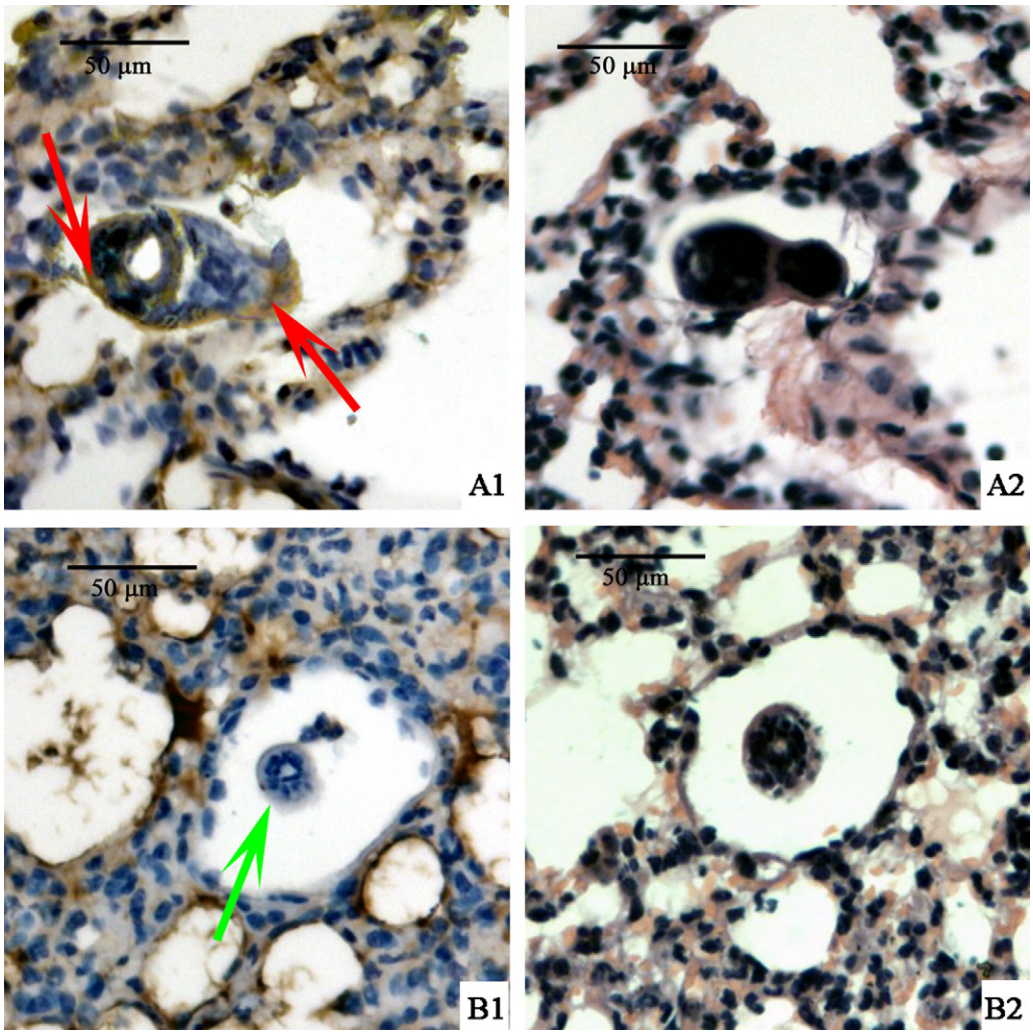

Fig. 3. Binding specificity of MppZL4 to lung-stage-schistosomula in mouse lung tissue analyzed by immunohistochemistry. Sections are shown in panels A1, A2 and B1, B2. In A1, reaction with MppZL4 showed a dark brown positive staining area surrounding the worm at the surface membrane (red arrow); while a dark blue signal was evident in the parenchyma. The worm in section B1 reacted with peptide M13KE phage, showing a yellow diffuse distribution in the mouse lung tissue and a dark blue staining of worm tissue (green arrow). Serial sections A2 and B2 were stained only with H&E, in order to locate the worm in the lung. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

whole adult worms of *S. japonicum*, including the oral and ventral suckers (Fig. 6C1, C2 and D; the fluorescence of the other parts of the surface of the parasites was as pronounced as for the oral and ventral suckers). In contrast, negative control adult did not show any significant fluorescence or showed minor fluorescence only around the oral and ventral suckers (Fig. 6E, shown by the green and the white arrows).

3.7. Lethal effects of MppZL4 and RhB-ZL4 on schistosomula in vitro

In order to evaluate any lethal effects of MppZL4 and RhB-ZL4 on schistosomula, worms were assigned to five groups and examined at 24, 48 and 72 h after separate culture *in vitro* with the different peptide: MppZL4, RhB-ZL4 as the experimental groups, M13KE, RhB-12P as the negative control groups and TBS as blank control group. After methylene blue staining, dead parasites had a dark blue and fuzzy structure, whereas viable worms were distinguished by a light blue color, translucent appearance and intact structure (Fig. S5). The experiment was repeated 3 times and the comparison of the 5 groups of the average mortality rate was shown (Table 4).

3.8. Deleterious effect of MppZL4 on schistosome development in mice

To gain an understanding of the effect of MppZL4 on schistosome growth and development, three discrete experiments were carried out as follows. The MppZL4, M13KE phage and TBS respectively were separately injected daily into the tail vein of mice at days 1–7 post-challenge with *S. japonicum*. Infected mice of one of the groups were treated with PZQ at day 36 post-challenge infection. All mice were euthanized at day 49 after challenge infection. The average worms burden, average worm weight per worm pair and LEPG (egg number in one gram liver tissue/mouse)/mouse were determined, and differences compared statistically (Table 5). As compared with infection control group or with the M13KE phage injection group, the worm burden, worm weight and LEPG from the mice treated with phage peptide ZL4 were significantly reduced. The liver egg load and adult worm weight were significantly reduced ($P < 0.01$).

4. Discussion

The schistosome tegument constitutes, along with the adult worm gut, the primary host–parasite interface. The tegument is

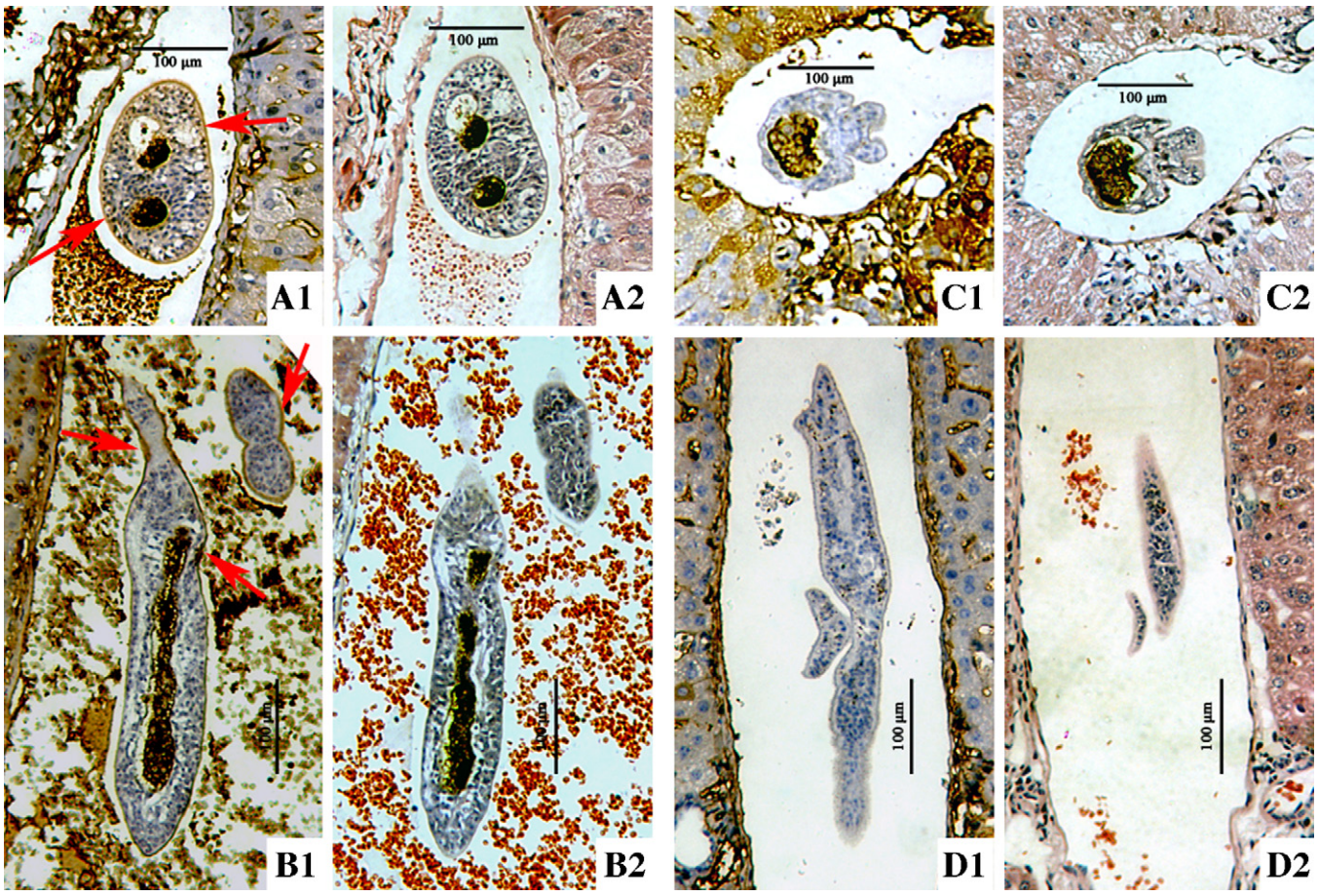

Fig. 4. Binding specificity of MppZL4 to liver-stage-schistosomula in mouse liver tissue by immunohistochemistry. The panels show sections of liver tissue of mice infected with *S. japonicum*. Panels A1, B1, C1 and D1 are immunohistochemical sections showing the structure of the parasite and mouse liver; panels A2, B2, C2 and D2 show sections stained with HE. Panels A1 and B1 illustrate reactivity with MppZL4, showing a dark brown positive reaction in the schistosomulum tegument (red arrow, surface membrane area in cross-section and longitudinal section of the worm) and in the mouse liver tissue (mainly red blood cells). Panels A2 and B2; a large number of red blood cells are evident in blood vessels around the schistosomula with the parasite gut clearly evident. Panels C1 and D1 showed reactivity with M13KE phage, revealing a negative reaction to the schistosomulum tegument, but with some positive reactivity in mouse liver tissue. The pronounced brown staining in the gut of the worm is due to host hemoglobin reacting with DAB (the chromogenic substrate used for the immunohistochemistry). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

syncytial and includes the outer plasma membrane and the basal membrane, both of which have two layers of tightly apposed elementary membranes [43]. In contrast to the blood stage forms, the outer plasma membrane of the cercarial tegument includes only a single layer of elementary membrane. This is covered by a glycocalyx, which is 0.5–1.0 μm thick. Formation of schistosomular tegument follows penetration by the cercaria into mammalian skin, during which time the tail also is shed from the cercaria. Within the mammalian host, the outer plasma membrane bilayer appears coincident with loss of the cercarial glycocalyx. Recent

Table 4
The killing effect of MppZL4 and RhB-ZL4 on *S. japonicum* schistosomula *in vitro*.

Group	Number of worms	Number of dead worms and mortality rate at different time points					
		24 h●		48 h▼		72 h▲	
		Mortality number	Mortality rate (%)[a]	Mortality number	Mortality rate (%)[b]	Mortality number	Mortality rate (%)[a]
I (TBS)	304 ± 5.08	53 ± 9.36	17.43	134 ± 10.29	44.08	245 ± 12.81	80.59
II (RhB-ZL4)	307 ± 4.81	108 ± 10.51	35.18	214 ± 12.56	69.71	307 ± 4.81	100[b]
III (RhB-12P)	299 ± 4.61	48 ± 8.28	16.05	115 ± 10.01	38.46	236 ± 11.53	78.93
IV (MppZL4)	301 ± 5.84	124 ± 10.51	41.20	208 ± 11.97	69.10	301 ± 5.84	100[b]
V (M13KE)	300 ± 5.28	43 ± 9.06	14.33	127 ± 9.86	42.33	228 ± 10.25	76.00

The data in the table are from the average of 3 trials. MppZL4 was from phage peptide ZL4 as the test group. RhB-ZL4 was from a synthetic peptide ZL4 with a Rhodamine B label. The TBS and M13KE groups were used as negative controls. The data were analyzed using χ^2-tests. The statistical comparison of the mean mortality rate between the groups is as follows: (a) I vs. II: ●$P = 0.000$; ▼$P = 0.000$; ▲$P = 0.000$. (b) I vs. III: ●$P = 0.650$; ▼$P = 0.161$; ▲$P = 0.611$. (c) I vs. IV: ●$P = 0.000$; ▼$P = 0.000$; ▲$P = 0.000$. (d) I vs. V: ●$P = 0.297$; ▼$P = 0.665$; ▲$P = 0.171$. (e) II vs. III: ●$P = 0.000$; ▼$P = 0.000$; ▲$P = 0.000$. (f) II vs. IV: ●$P = 0.127$; ▼$P = 0.872$; ▲P^{**}. (g) II vs. V: ●$P = 0.000$; ▼$P = 0.000$; ▲$P = 0.000$. (h) III vs. IV: ●$P = 0.000$; ▼$P = 0.000$; ▲$P = 0.000$. (i) III vs. V: ●$P = 0.558$; ▼$P = 0.334$; ▲$P = 0.391$. (j) IV vs. V: ●$P = 0.000$; ▼$P = 0.000$; ▲$P = 0.000$. There were no significant differences between groups II and IV in the three time points after 24 h. There were highly significant differences between groups II and. III, IV and V at 24 h, 48 h and 72 h.

[a] Mortality rate = the number of dead worms in each sample at each time point/the total number worms in each sample at each time point.

[b] On at least one case, the value of the weight variable was zero, negative, or missing. Such cases are invisible to statistical procedures and graphs which need positively weighted cases, but remain on the file and are processed by non-statistical facilities such as LIST and SAVE.

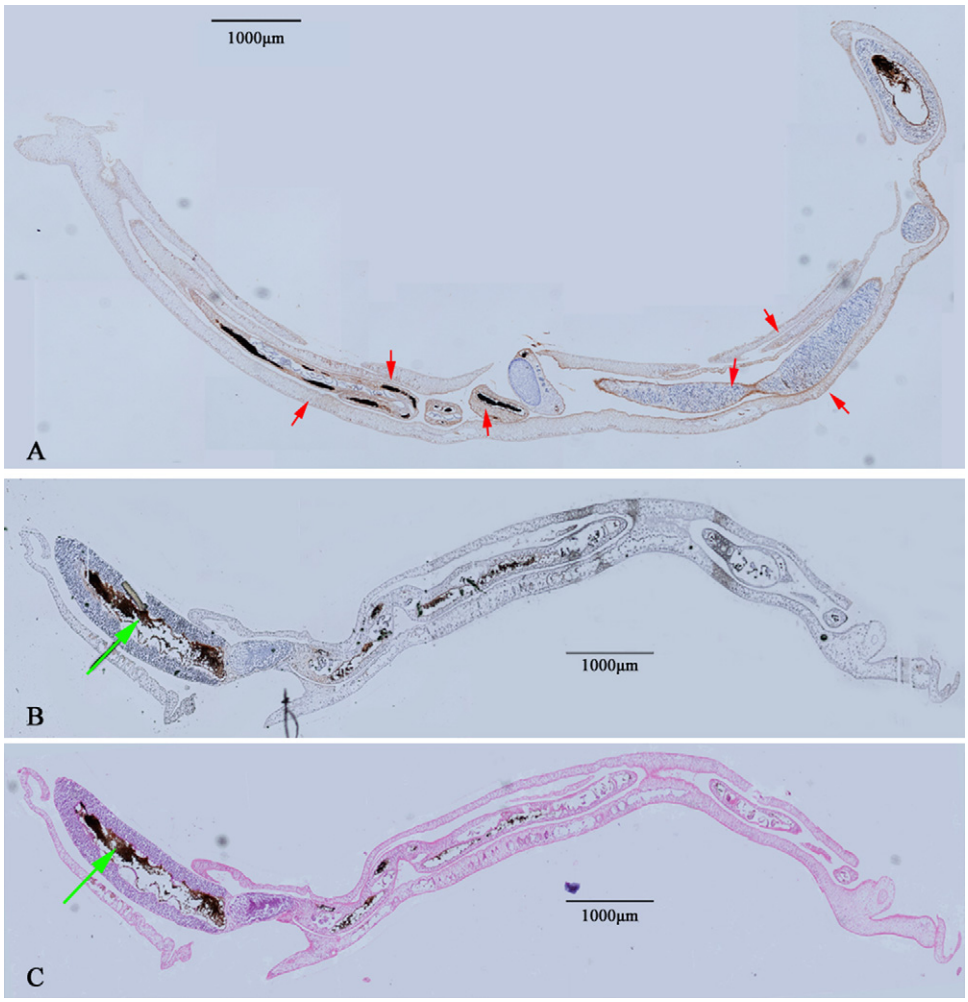

Fig. 5. Binding specificity of MppZL4 to *Sj* adult worms by immunohistochemistry. Panel A is a section of an adult male and female worm pair and reactivity with MppZL4 showing a dark brown positive reaction evident in the surface tegumentary membrane, especially in the female (the red arrows). The pronounced brown staining in the gut is due to host hemoglobin reacting with DAB (the chromogenic substrate used in the immunohistochemical analysis). Panel B shows a section of the male and female worm pair probed with the control M13KE phage; no reactivity was observed except the staining in the gut (the green arrow). C shows a section of the adult male and female worm pair stained with H&E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

findings have revealed differential expression of lactate and glucose transporters, SmSLP, the *S. mansoni* stathmin-like protein [44], SmAQP – *S. mansoni* aquaporin [45,46], and other proteins at the surface of schistosomula, but not cercariae [47]. In addition to differences between the tegument of these two developmental stages, an expanding number of receptors are known from the surface of blood stages of schistosomes including signal transduction components, transporters, ion channels and so forth [12–16,47–50]. In the present study, we explored whether heterologous peptides might exhibit affinity for the surface of blood stages of *S. japonicum*, if so, whether they might promote and/or antagonize parasite growth and development. Consequently, we used phage display technology to identify a MppZL4, NH_2-YSGLQDSSLRLR-C, that binds strongly to mammalian stages of the schistosome, but not to cercariae, and which displays potent anti-schistosomal activity.

The phage display technique has been investigated previously to search for *S. japonicum*-binding peptides. These studies have identified epitopes of schistosome antigens recognized by monoclonal or polyclonal antibodies raised against schistosomes or in serum from non-permissive hosts including *M. fortis*. This information is being

Table 5
Effect of phage peptide ZL4 on schistosome development *in vivo*.

Group	Worm Burden recovery (mean ± SD)	Worm burden reduction rate● (%)	Worm weight (mean ± SD) (mg)	Worm weight reduction rate▾ (%)	LEPG (mean ± SD)	LEPG reduction rate▲ (%)
I (PZQ)	11.45 ± 5.32	48.17	1.38 ± 0.38	45.88	2436.4 ± 925.5	85.84
II (ZL4)	16.39 ± 5.68	25.80	1.68 ± 0.49	34.12	7011.1 ± 3655.9	59.24
III (M13KE)	21.73 ± 8.32	1.63	2.23 ± 0.46	12.55	15381.8 ± 3789.9	10.57
IV (PBS)	22.09 ± 5.09	–	2.55 ± 0.47	–	17200.0 ± 4448.6	–

Data in the table were the average from three separate experiments. Thirty mice were used in each of the groups I–IV. ●, worm burden reduction; ▾ worm weight reduction; and ▲ LEPG reduction compared to IV group (infection control group). The results of statistical comparison between the groups are as follows: (a) I vs. IV: ●$P = 0.000$; ▾$P = 0.000$; ▲$P = 0.000$. (b) II vs. IV: ●$P = 0.000$; ▾$P = 0.000$; ▲$P = 0.000$. (c) III vs. IV: ●$P = 0.068$; ▾$P = 0.090$; ▲$P = 0.057$. (d) I vs. II: ●$P = 0.054$; ▾$P = 0.071$; ▲$P = 0.000$. (e) I vs. III: ●$P = 0.000$; ▾$P = 0.000$; ▲$P = 0.000$. (f) II vs. III: ●$P = 0.000$; ▾$P = 0.000$; ▲$P = 0.000$.

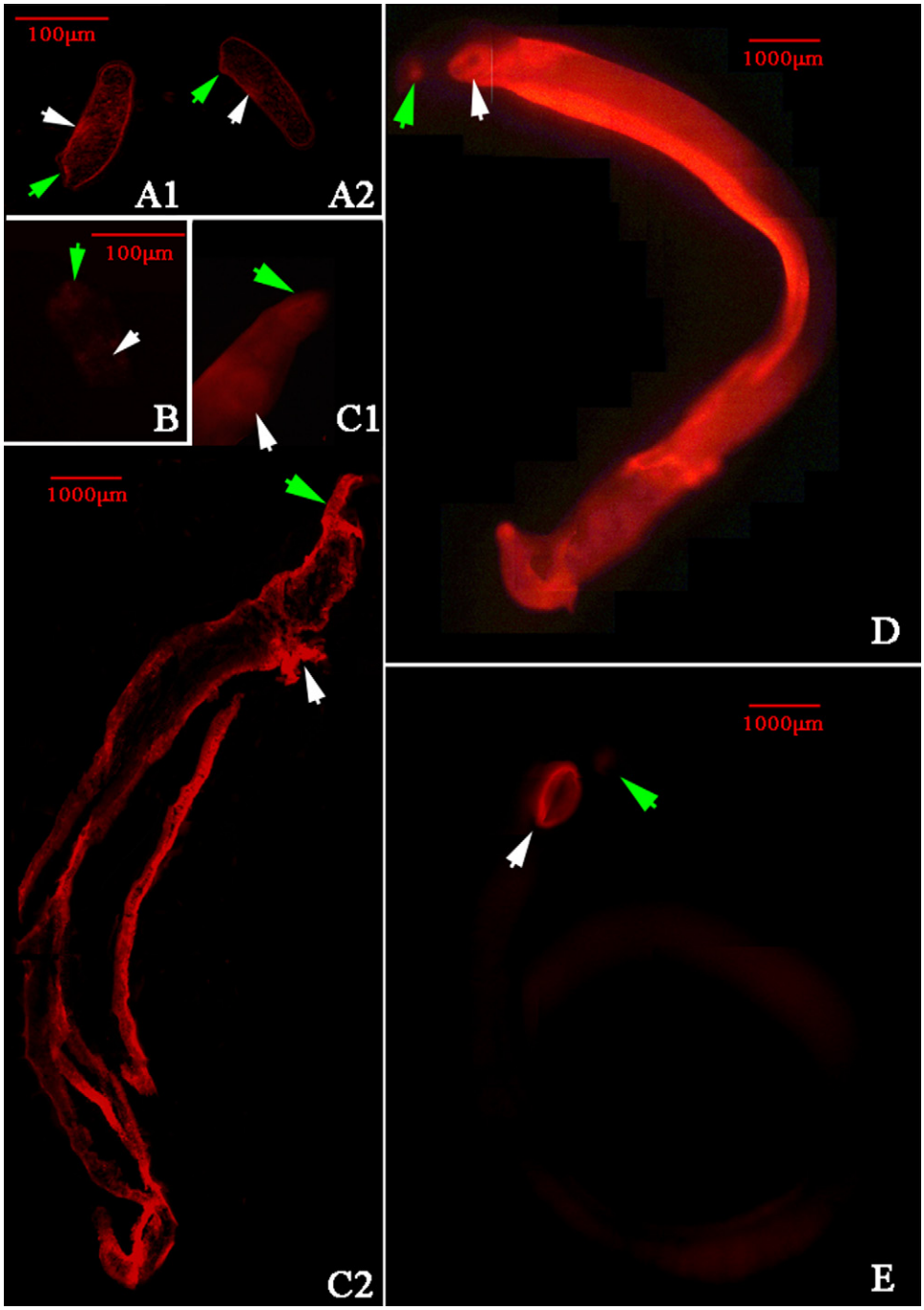

Fig. 6. Binding specificity of RhB-ZL4 to *S. japonicum* schistosomula *in vitro* and adult worms *in vivo*. RhB-ZL4 was incubated with schistosomula and cercariae while RhB-12P was incubated with schistosomula and cercariae as negative control. The results were observed using a fluorescence microscope. As is evident, RhB-ZL4 bound to the schistosomula on the surface of the tegument, including the regions of the oral and ventral suckers (panels A1and A2, the green arrow denotes the oral sucker and the white arrow denotes the ventral sucker). In contrast, RhB-ZL4 did not bind cercariae (not shown), whereas RhB-12P did not bind schistosomula. A small amount of red fluorescence was observed on the oral sucker and ventral sucker of the schistosomula binding RhB-12P (panel B, the green and the white arrows). Panels C1 (only the oral and ventral sucker), D and E show adult worms which were directly observed by fluorescence microscopy, and C2 was a frozen section of C1. Adult worms of *S. japonicum* from the I group of mice which were injected with RhP-ZL4 showed fluorescence signals along the tegument (panel C2) of the whole worm, the fluorescence of the other parts of the surface of the parasite was as pronounced as on the oral and ventral suckers (panels C1, C2 and D), while negative control adults did not show significant fluorescence or showed fluorescence only around the oral and ventral suckers (panel E). The green arrows indicate the oral sucker while the white arrows indicate the ventral sucker. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

employed to guide development of vaccines or new diagnostics [51–56].

Here we focused on phage display peptide comparisons between cercariae and mechanically transformed schistosomula since neither of these stages would be contaminated with mammalian host cell factors. The tegumental ultrastructure of mechanically transformed schistosomula is basically similar to that of schistosomula at 3 h post-penetration of mammalian skin; early ultrastructural changes of schistosomula transformed from cercariae occur primarily in the body wall and the secretory glands of *S. japonicum* [23]. We employed a reverse differential biopanning technique to search for phage display peptides which bind

schistosomula but not cercaria. The initial step involved adsorption of the peptide library to cercariae to eliminate cercaria-specific peptides. Second, schistosomula were incubated with phage peptides that did not adhere to cercariae. After performing three cycles of reverse absorption and bio-panning, numerous phages were obtained from which 15 positive phage peptides were randomly picked for DNA sequencing; three peptides were encoded by several of these clones, namely peptide ZL1 (the insert amino acid sequence DYPSANKWPRYV), ZL4 and ZL6 (the insert amino acid sequence DRYSEIRTSSTL).

We undertook further investigation of the specific binding capacity and potential functional role of these peptides in regard to the schistosomula tegument. Only MppZL4 bound strongly to the parasite and, accordingly, our subsequent investigation focused on this peptide. Immunohistochemical localization on developmental stages revealed that MppZL4 bound to the tegument of newly transformed schistosomula, lung schistosomula, liver schistosomula and adult worms but not to cercariae. There are several reasons for the background staining in the mouse tissue. First, In order to locate the worms in the lung and liver tissues, the animals were not perfused to avoid washing away the parasites. This resulted in host red blood cells remaining in the vasculature which contained peroxidase and thus showed positive results on immunohistochemistry. Second, the samples were collected after mice had been injected with MppZL4 15 min and the phage which had been ingested by macrophages and other cells of the reticuloendothelial system had not been completely metabolized [57–59].

At the same time, RhB-ZL4, the Rhodamine B-conjugated peptide ZL4, was able to bind to the tegument of newly transformed schistosomula and adult worms as well. To our knowledge, this is the first report of an exogenous molecule having specific developmental stage-binding characteristics. However, the mechanism of its binding remains to be determined. The peptide ZL4 has a theoretical p*I* of 8.75, and according to this characteristic, the binding of peptide ZL4 with the schistosome tegument might be related to the difference in charge deviation (electric charge gradient).

Finally, ZL4 exhibited potent schistosomicidal activity *in vitro*. Moreover, ZL4 interfered and negatively effected the growth, development and fecundity of *S. japonicum in vivo*. As with the mechanism of surface binding, how peptide ZL4 exerts its detrimental effects on schistosomes is not yet clear; but these findings clearly warrant further investigation. We anticipate that understanding how the ZL4 peptide binds to the schistosomulum surface and how it exerts its anti-schistosomal action could provide leads to new drugs or other interventions for schistosomiasis. We suggest also that ectogenic polypeptides, which can bind to the tegument of *S. japonicum*, might be adapted as vectors to deliver experimental probes and/or pharmaco-effector compounds to the schistosome tegument, including drugs and immunological mediators.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2011.09.001.

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