

# Peptide-Based Subunit Vaccine against Hookworm Infection

Mariusz Skwarczynski<sup>1</sup>\*, Annette M. Dougall<sup>2</sup>\*, Makan Khoshnejad<sup>1</sup>, Saranya Chandrudu<sup>1</sup>, Mark S. Pearson<sup>2</sup>, Alex Loukas<sup>2\*</sup>, Istvan Toth<sup>1,3\*</sup>

**1** The University of Queensland, School of Chemistry and Molecular Biosciences, St. Lucia, Queensland, Australia, **2** Centre for Biodiscovery and Molecular Development of Therapeutics, Queensland Tropical Health Alliance, James Cook University, Cairns, Queensland, Australia, **3** The University of Queensland, School of Pharmacy, Woolloongabba, Queensland, Australia

## Abstract

Hookworms infect more people than HIV and malaria combined, predominantly in third world countries. Treatment of infection with chemotherapy can have limited efficacy and re-infections after treatment are common. Heavy infection often leads to debilitating diseases. All these factors suggest an urgent need for development of vaccine. In an attempt to develop a vaccine targeting the major human hookworm, *Necator americanus*, a B-cell peptide epitope was chosen from the apical enzyme in the hemoglobin digestion cascade, the aspartic protease *Na*-APR-1. The A<sub>291</sub>Y alpha helical epitope is known to induce neutralizing antibodies that inhibit the enzymatic activity of *Na*-APR-1, thus reducing the capacity for hookworms to digest hemoglobin and obtain nutrients. A<sub>291</sub>Y was engineered such that it was flanked on both termini by a coil-promoting sequence to maintain native conformation, and subsequently incorporated into a Lipid Core Peptide (LCP) self-adjuncting system. While A<sub>291</sub>Y alone or the chimeric epitope with or without Freund's adjuvants induced negligible IgG responses, the LCP construct incorporating the chimeric peptide induced a strong IgG response in mice. Antibodies produced were able to bind to and completely inhibit the enzymatic activity of *Na*-APR-1. The results presented show that the new chimeric LCP construct can induce effective enzyme-neutralising antibodies in mice, without the help of any additional toxic adjuvants. This approach offers promise for the development of vaccines against helminth parasites of humans and their livestock and companion animals.

**Citation:** Skwarczynski M, Dougall AM, Khoshnejad M, Chandrudu S, Pearson MS, et al. (2012) Peptide-Based Subunit Vaccine against Hookworm Infection. PLoS ONE 7(10): e46870. doi:10.1371/journal.pone.0046870

**Editor:** David Joseph Diemert, The George Washington University Medical Center, United States of America

**Received:** June 20, 2012; **Accepted:** September 6, 2012; **Published:** October 3, 2012

**Copyright:** © 2012 Skwarczynski et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by the National Health and Medical Research Council of Australia (NHMRC Programme Grant 496600), <http://www.nhmrc.gov.au/>. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors also thank Thalia Guerin for her correction of this manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: alex.loukas@jcu.edu.au (AL); i.toth@uq.edu.au (IT)

† These authors contributed equally to this work.

## Introduction

Hookworm infection causes one of the world's most debilitating neglected tropical diseases. The human hookworm (*Necator americanus*) infects over 700 million people worldwide, predominantly in indigent rural and tropical regions [1]. Chronic infection results in long-term pathological consequences primarily due to ongoing intestinal blood loss resulting from the feeding activities of these hematophagous parasites. Heavy infection leads to iron-deficiency anemia and can manifest as impaired neurological and intellectual functioning in children, reduced work capacity in adults, and severe adverse outcomes in pregnancy [2]. Those most vulnerable to the harmful effects of hookworm include children and pregnant women, who are unable to tolerate the chronic blood loss and iron deficiency anemia due to their lower iron reserves [3]. These factors have significant influence on current and future productivity and economic well being of infected populations. Benzimidazole drugs are commonly used for the treatment and eliminate adult parasites. However, chemotherapy has limited efficacy and reinfection after treatment is common [4,5]. The problems with drug effectiveness and the looming threat of drug resistance suggest that alternatives to mass drug

administration are urgently needed. Development of a vaccine that would prevent the acquisition of moderate or heavy intensity hookworm infection would be a major advance in reducing the morbidity caused by this parasite [6,7]. Currently, there is no human hookworm vaccine on the market or in advanced clinical trials.

Hookworms obtain their nourishment primarily by ingesting blood and digesting the hemoglobin and serum proteins released from lysed erythrocytes. *Na*-APR-1 is a cathepsin D aspartic protease derived from the gut of adult *N. americanus* where it initiates the hemoglobin digestive cascade [8,9]. Therefore, blocking the catalytic activity of *Na*-APR-1 via the induction of neutralizing antibodies should result in starving and ultimately killing of the parasite. Indeed, it was demonstrated that APR-1 could be used as an efficacious hookworm vaccine antigen against *A. caninum* in dogs. Vaccination with the recombinant *N. americanus* or *A. caninum* enzymes induced antibodies that bound to the gut of the parasite and neutralized the enzymatic activity of the protease *in vitro*. When vaccinated dogs were then challenged with hookworm larvae they had significantly diminished adult parasite burdens and a reduction in blood loss was observed [8,10].

However, production of APR-1 at a commercial scale has proven to be challenging due to protein aggregation and low manufacturing yield obtained from eukaryotic expression systems. Previously, the immunogenic peptide epitope from *N. americanus* APR-1 (A<sub>291</sub>Y, AGPKAQVEAIQKY) was shown to induce the production of neutralizing antibodies *in vivo*. These antibodies were able to inhibit enzymatic activity of APR-1 against synthetic peptide and natural protein substrates [11].

Peptide antigens are not immunogenic by themselves and require appropriate delivery systems and strong, often toxic, adjuvants to stimulate desired immune responses [12]. For example, in the above-mentioned study the use of toxic complete Freund's adjuvant composed of inactivated and dried mycobacteria was necessary to stimulate immune responses against the A<sub>291</sub>Y epitope. To avoid this problem, lipidation of peptides emerged as a promising strategy for delivery of peptide subunit vaccines. The self-adjuvanting Lipid Core Peptide (LCP) delivery system has a demonstrated ability to induce strong immune responses against the incorporated peptide-epitopes without the help of external adjuvants, and is considered to be a capable platform for development of human vaccines [13,14].

In this paper, we have designed, synthesized and characterized a short series of A<sub>291</sub>Y conjugates. We have used the coil-promoting sequence from the yeast GCN4 protein to induce native helical A<sub>291</sub>Y epitope conformation [11,13,15,16]. Subsequently, chimeric and parent A<sub>291</sub>Y epitopes were incorporated into the LCP delivery system. The various LCP constructs induced variable IgG responses in mice, nonetheless these antibodies were able to bind to and completely inhibit the enzymatic activity of *N*-APR-1. The results presented show that the new chimeric epitope can be presented as an LCP construct to induce an effective enzyme-neutralizing response without the help of any additional adjuvants and delivery systems. This approach offers promise for the development of vaccines against helminth parasites of humans and their livestock and companion animals.

## Materials and Methods

### I. Materials

Protected L-amino acids were obtained from Novabiochem (Laufelfingen, Switzerland) and Mimotopes (Melbourne, Australia). pMBHA resin was purchased from Peptides International Inc. (Kentucky, USA). 1-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Mimotopes. HPLC grade acetonitrile and *N,N*-dimethylformamide were obtained from Ajax Finechem (Sydney, Australia). Trifluoroacetic acid (TFA) was obtained from Merck (Kilsyth, Australia). All other reagents were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Microwave assisted Fmoc SPPS was carried out by using a SPS mode on CEM Discovery reactor (CME Corporation, Matthews, NC, USA). An AKel-F HF apparatus (Peptide Institute, Osaka, Japan) was used for HF cleavage. ESI-MS was performed on a Perkin-Elmer-Sciex API3000 instrument with Analyst 1.4 software (Applied Biosystems/MDS Sciex, Toronto, Canada). Analytical RP-HPLC was performed on an Agilent instrument with a 1.0 mL/min flow rate and detection at 214 nm. Separation was achieved by running gradient mode of 0–100% solvent B over 40 min with solvent A (0.1% TFA/H<sub>2</sub>O) and two kind of solvent B (B1:90% MeCN/0.1% TFA/H<sub>2</sub>O or B2:90% MeOH/0.1% TFA/H<sub>2</sub>O) on either a Vydac analytical C4 column (214TP54; 10 μm, 4.6×250 mm) or a Vydac analytical C18 column (218TP54; 10 μm, 4.6×250 mm). Purification was carried out by preparative RP-HPLC using a Waters Delta 600 system with a 10.0 mL/min flow rate. Compounds were detected at 230 nm.

Separations were performed with solvent A and solvent B1 on either a Vydac preparative C4 column (214TP1022; 10 mm, 22×250 mm) or a Vydac preparative C18 column (218TP1022; 10 mm, 22×250 mm). CD spectra were measured on a JASCO J-710 spectropolarimeter (Tokyo, Japan) using a quartz cuvette of 1 mm path length at 23°C. The CD spectra were measured in water with trifluoroethanol (TFE) at concentration of 10% (v/v).

### II. Synthesis of Peptide Epitopes and LCPs

Peptides **1** and **2** (Figure 1) were synthesized using manual SPPS at 0.1 mmol scale with pMBHA (0.45 mmol NH<sub>2</sub>/g) resin. The synthesis was carried out as previously reported [17,18,19]. Shortly thereafter, Boc-deprotection was performed with neat TFA (2×1 min), followed by a 1 min DMF flow wash, and a 30 min coupling with pre-activated Boc-protected amino acid (1 hour in the case of lipoamino acid (2-(*R/S*)-[(tert-butoxycarbonyl)amino]-dodecanoic acid)) [17,20]. Amino acid pre-activation was achieved by dissolving amino acids (4.2 equiv.) in 0.5 M HBTU/DMF solution (4.0 equiv.), and DIPEA (6.2 equiv.). Activation proceeded for 2–3 min, except for lipoamino acids which were pre-activated for 5–10 min. Double coupling was performed for all amino acids.

**Peptide 1.** Yield: 33%. Molecular Weight: 1443.7. [M+1H]<sup>+</sup> m/z 1444.7 (calc. 1444.77), [M+2H]<sup>2+</sup> m/z 722.8 (calc. 722.89). t<sub>R</sub> = 13.79 (0–100% Solvent B1, 30 min; C18), t<sub>R</sub> = 21.37 (0–100% Solvent B2, 30 min; C18).

**Peptide 2.** Yield: 28%. Molecular Weight: 3210.7. [M+3H]<sup>3+</sup> m/z 1071.8 (calc. 1071.25), [M+4H]<sup>4+</sup> m/z 803.9 (calc. 803.69). t<sub>R</sub> = 13.8 (0–100% Solvent B1, 30 min; C18), t<sub>R</sub> = 21.33 (0–100% Solvent B2, 30 min; C18).

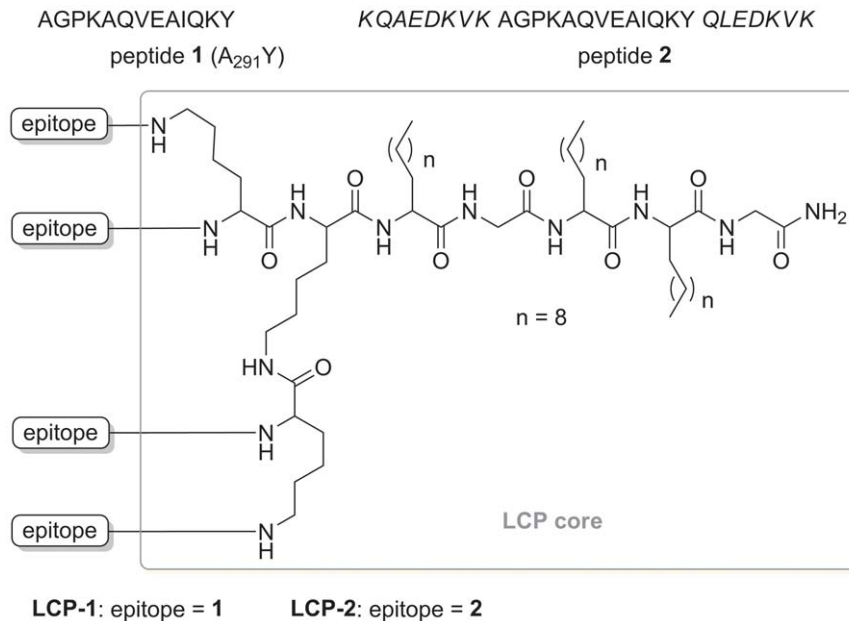
**LCP-1** and **LCP-2** (Figure 1) were synthesized using microwave-assisted SPPS at 0.05 mmol scale with pMBHA (0.45 mmol NH<sub>2</sub>/g) resin. Microwave-assisted SPPS was applied with two five minute microwave-assisted couplings (SPS mode, power 20 W, temperature 70°C, ΔT = 1°C) for each amino acids (4.2 equiv.) using HBTU (4.0 equiv.) and DIPEA (6.2 equiv.) [21]. The lipoamino acids were coupled in the same manner as regular amino acids but with extended (5–10 mins) HBTU/DIPEA pre-activation. N-terminal acetylation, TFA-assisted Boc cleavage and final cleavage from resin was performed as reported previously [17].

**LCP-1.** Yield: 46%. Molecular Weight: 6814.1. [M+5H]<sup>5+</sup> m/z 1364.3 (calc. 1363.81), [M+6H]<sup>6+</sup> m/z 1136.8 (calc. 1136.67). t<sub>R</sub> = 20.7, 20.96, 21.19, 21.66 (0–100% Solvent B1, 30 min; C4), t<sub>R</sub> = 29.82, 30.39, 30.61, 31.03 (0–100% Solvent B2, 30 min; C4).

**LCP-2.** Yield: 38%. Molecular Weight: 13886.1. [M+11H]<sup>11+</sup> m/z 1263.1 (calc. 1263.38), [M+12H]<sup>12+</sup> m/z 1158.4 (calc. 1158.11). t<sub>R</sub> = 18.39, 18.61 (0–100% Solvent B1, 30 min; C4), t<sub>R</sub> = 27.20 (0–100% Solvent B2, 30 min; C4).

### II. Toxicological Evaluation

**Hemolytic assay.** Using a standard hemolytic assay, the capacity of LCP compounds (**LCP-1** and **LCP-2**) to induce hemolysis was examined (Figure 2). Blood was collected from a healthy human volunteer with written informed consent (protocol approved by the University of Queensland Ethics Committee, approval number 2009000661). The toxicity of the compounds was tested in three concentrations, (10, 50, and 100 μM) and incubated at 37°C for one hour. SDS was used as the positive control and PBS as negative control. After one hour, the plate was centrifuged at 750×g for 15 min and 75 μL of supernatant per well was transferred to a new 96 well plate. The absorbance at 540 nm was recorded by UV spectrometer. The



**Figure 1. The structure of peptide epitopes (1, 2) and LCP constructs (LCP-1 and LCP-2).** Peptide epitope 2 retains native epitope sequence 1 (regular fonts) flanked by helicity inducing sequence from GCN4 protein (italic fonts). doi:10.1371/journal.pone.0046870.g001

data was calculated according to a standard formula:

$$\% \text{Hemolysis} = [(A_{540} - \text{min}A_{540}) / (\text{max}A_{540} - \text{min}A_{540})] \times 100\%$$

where:

- $-A_{540}$  is the average absorption of compound at 540 nm
- $-\text{min}A_{540}$  is the average absorption of PBS
- $-\text{max}A_{540}$  is the average absorption of SDS.

**MTT assay.** The toxicity of the compounds (1–4) was examined by MTT assay (Figure 2). The Caco-2 cells were cultivated in a flask with DMEM (Dulbecco's Modified Eagle Medium) to reach 80% confluence. The cells were split into a 96 well plate (100  $\mu\text{L}$  of cells per well) prior to the MTT test (to let the cells adhere to on the surface). The compounds 1–4 were prepared in three concentrations of 10, 50, and 100  $\mu\text{M}$  in PBS. The culture medium was removed and 100  $\mu\text{L}$  of the compound solutions were added to each well. The cells were incubated for 24 hours. After the incubation, the solutions were removed and 20  $\mu\text{L}$  of MTT followed by 80  $\mu\text{L}$  of DMEM medium was added to each well. The plates were incubated for 4 hours, centrifuged at  $750 \times g$  for 5 mins, and the supernatant discarded. Fifty microliters of DMSO was added to each well to dissolve the purple crystals. The UV absorbance readings were taken at 570 nm wavelength. PBS was used as a blank and SDS, 100% as a negative control. The data was calculated according to a standard formula:

$$\% \text{Viability} = [(A_{540}) / (\text{max}A_{540})] \times 100\%$$

where:

- $A_{540}$  is the average absorption of compound at 540 nm
- $\text{max}A_{540}$  is the average absorption of DMEM (positive control).

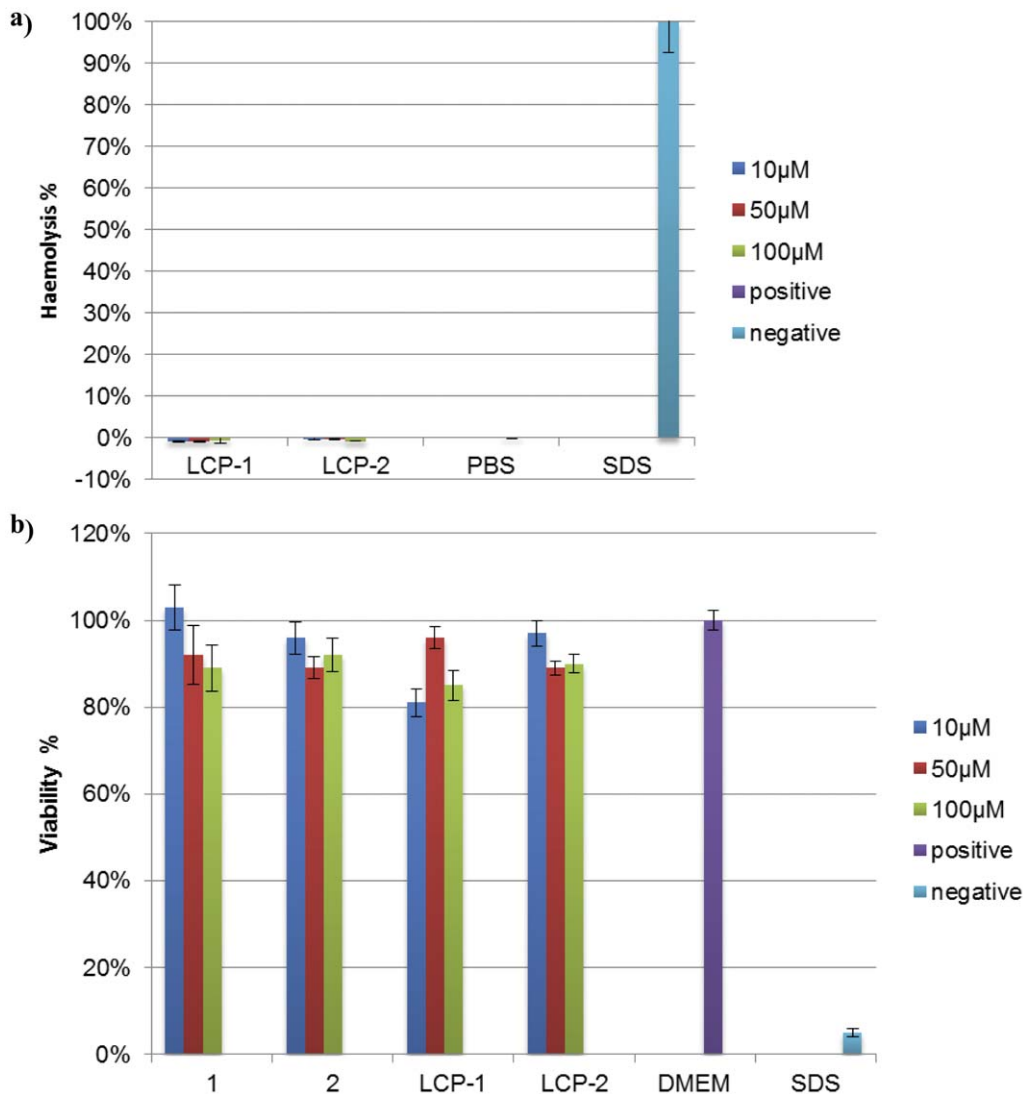
### III. Intraperitoneal Immunization of Mice with Constructs

Groups of four female BALB/c mice (4–6 weeks old) were intraperitoneally injected with 30  $\mu\text{g}$  of peptide 1+Freund's adjuvants, peptide 2+Freund's adjuvants, LCP-1 or LCP-2 in 200  $\mu\text{L}$  of PBS. All animal protocols used were approved by the James Cook University Ethics Committee (A1484) in accordance with National Health and Medical Research Council (NHMRC) of Australia guidelines. For the Freund's adjuvants groups, peptides 1 and 2, 100  $\mu\text{L}$  (30  $\mu\text{g}$ ) of construct was mixed with an equal volumes of Freund's complete adjuvant (CFA) for the first immunization and incomplete adjuvant for the subsequent immunizations (Sigma). Mice were injected 4 times on days 0, 21, 33, and 43. Mice were euthanized on day 70 and the blood collected via cardiac puncture. Sera were separated from clotted blood by centrifugation at 3 000 rpm for 10 min.

In a second experiment, a total of five female BALB/c mice were immunised with 60  $\mu\text{g}$  per immunization of LCP-2 on days 0, 21, 32, and 35. On day 42 mice were euthanized and the sera collected as above.

### IV. ELISA

Antibody responses to the constructs were measured by ELISA. Peptides or LCPs were coated on 96 well flat bottom ELISA plates (BD) at a concentration of 5  $\mu\text{g}/\text{mL}$  in 50 mM sodium carbonate buffer at pH 9.6 and incubated overnight at 4°C. Non-reactive sites were blocked with 3% bovine serum albumin in PBS/0.05% Tween 20 (Sigma) for 1 hr at RT. Individual mouse sera were added in duplicate using 10-fold serial dilutions to plates that were coated with the respective immunogens. Total IgG binding was detected using 1:2000 sheep anti-mouse IgG (H&L) conjugated to horseradish peroxidase (Chemicon). Bound anti-mouse IgG was detected using TMB single solution (Invitrogen). Plates were measured at 655 nm on a POLARstar Omega microplate reader (BMG Labtek). Absorbance was measured at 655 nm and corrected for background using wells that received sera (1:100) in the absence of peptide or LCP bound to the plate.



**Figure 2. Toxicity of lipid core peptides LCP-1 and LCP-2.** (a) Haemolytic potential of lipid core peptides (LCP) was measured by comparing the absorbance (540 nm) of blood samples incubated with the LCP vaccine candidates with that of samples incubated with a positive control (SDS, 100% haemolysis) and a negative control (PBS, 0%). Mean and SD of triplicates samples shown. (b) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assays in triplicate were performed on the Caco-2 cell line with varying concentrations of compounds (10–100 μM). doi:10.1371/journal.pone.0046870.g002

## V. Enzyme Neutralisation Assay

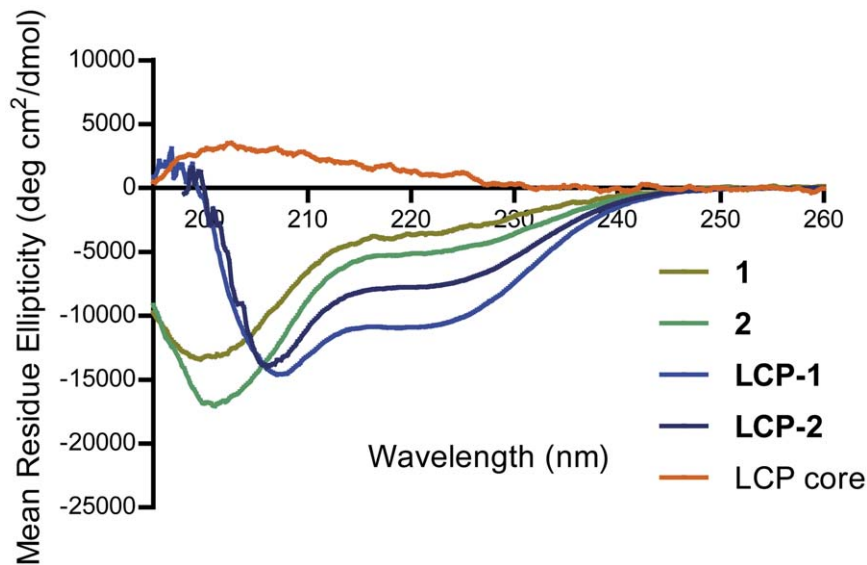
The IgG from the two highest responding mice (experiment 2) were bound to protein G sepharose (Millipore) and eluted as previously described [8,11]. After purification, IgG was concentrated and buffer exchanged into PBS using Nanosep<sup>®</sup> centrifugal devices (Pall) as per the manufacturer's protocol. Yeast derived recombinant of *Nα*-APR-1 (60 ng) (kindly provided by Dr Bin Zhan, George Washington University) was incubated with 2.5 and 1.25 μg of purified IgG in 50 mmol/L sodium acetate. Reactions were performed in black 384 well plates (Greiner Bio One) in 50 μl; the substrate 7-methoxycoumarin-4-acetyl-GKPIFFRLK(DNP)-d-Arg-amide (MoCAc-GKPIFFRLK) (Sigma) was added to a final concentration of 1.0 mmol/L, and the fluorescence generated (relative to substrate in buffer alone) by substrate hydrolysis was measured as described [8] using a POLARstar Omega microplate reader. Inhibition of enzymatic activity was analysed as a percentage of the fluorescence generated

from an equivalent reaction containing equivalent amounts of control IgG (Millipore) at 2.5 μg and 1.25 μg.

## Results

### I. Synthesis and Characterization

All compounds were synthesized using the stepwise Boc-SPPS method in a similar manner to that described previously [17]. The peptide epitopes and the tetra-branched LCP constructs were obtained in highly pure form without difficulties. The CD spectra were measured in water with trifluoroethanol (TFE) because this solvent is known to stabilize the  $\alpha$ -helical structure in peptides and is often used to induce the native structure of protein fragments [22,23,24]. According to CD analysis peptide epitopes showed random coil rather than helical conformation with the minimum observed at 199 nm and 201 nm for peptides **1** and **2**, respectively, while LCP core alone clearly did not form helical structure (Figure 3). In contrast, **LCP-1** and **LCP-2** showed



**Figure 3. CD spectra of peptides 1, 2, LCP-1 and LCP-2 in the presence of 10% of TFE.** Spectra were acquired at 1 nm intervals from 260 nm to 190 nm, were the average of 3 individual scan and are reported in units of mean residue ellipticity [0]. doi:10.1371/journal.pone.0046870.g003

helical conformation, with double minima at 207 nm, 219 nm and 206 nm, 220 nm.

## II. Toxicological Evaluation

Basic toxicological evaluation of all compounds was performed using MTT and haemolytic assays (Figure 2). It was clearly demonstrated that all compounds were non-toxic to Caco-2 cells. Lipophilic compounds, **LCP-1** and **LCP-2** were also assessed for toxicity to red blood cells, and were shown to be non-haemolytic, even at high concentrations (100  $\mu$ M).

## III. Antibody Binding and Neutralization of Recombinant *Na*-APR-1

Mice that were immunized with peptide **1** with Freund's adjuvant did not induce a detectable IgG response in any mice. Peptide **2** did not induce an IgG response alone but did induce a weak IgG response when administered with adjuvant. **LCP-1** ( $A_{291}Y$ -LCP) induced a very weak IgG response that was barely detectable, however **LCP-2** ( $A_{291}Y$ -GCN4-LCP) induced a stronger IgG response but the titer was variable between individual mice (Figure 4a). We repeated the vaccination experiment with **LCP-2** (experiment 2) this time using 60  $\mu$ g of LCP-4 in an attempt to improve the consistency and titer of the antibody response (Figure 4b). Increasing the quantity of **LCP-2** for immunization resulted in a more consistent antibody response between mice and higher titer responses.

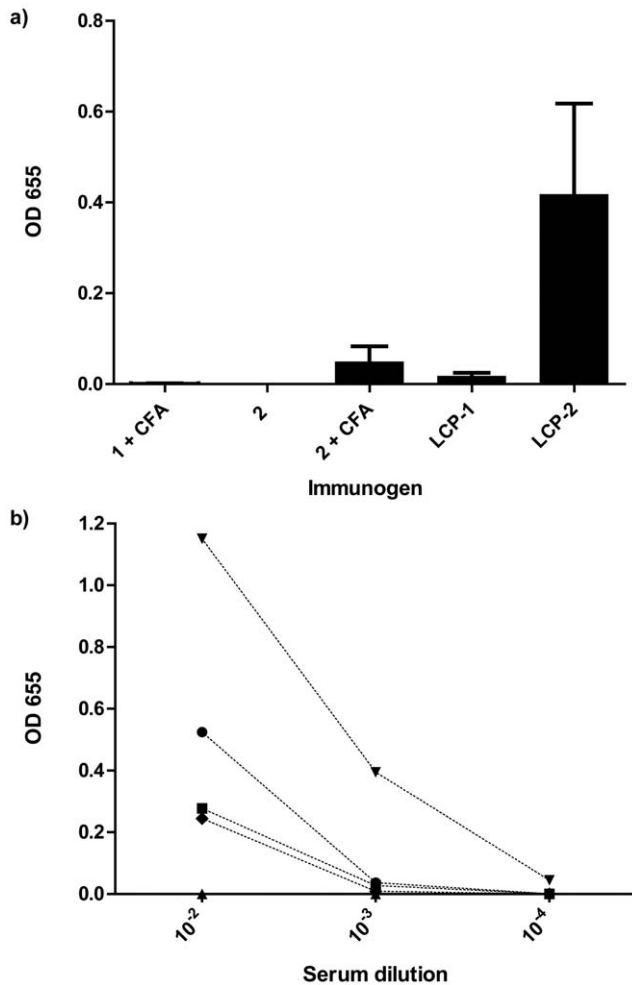
IgG was purified from the two highest responding mice (Figure 4b) that received the 60  $\mu$ g dose of **LCP-2** and used in an *Na*-APR-1 enzymatic neutralization assay. IgG (2.5  $\mu$ g) from both mice inhibited the ability of APR-1 to digest a fluorescent peptide substrate by 92–100% compared to control IgGs. The inhibition is dose-dependent (Table 1) as described previously with polyclonal and monoclonal IgGs from mice immunized with recombinant *Na*-APR-1 [8,11].

## Discussion

In our attempts to develop a human hookworm vaccine we identified a number of antigens that showed promise in animal

models [6]. One of the lead antigens derived from the blood-feeding adult stage of the parasite was *Na*-APR-1 [8,10]. There are numerous obstacles facing the production and safe use of a vaccine based on an active protease, so we identified an epitope from *Na*-APR-1,  $A_{291}Y$ , that was capable of inducing antibodies that neutralized the catalytic activity of the recombinant enzyme [11]. Here we describe a self-adjuvanting approach to produce  $A_{291}Y$  fused to GCN4 and LCP, and show that antibodies generated to the construct can neutralize the enzymatic activity of the native enzyme, highlighting the utility of this approach for the development of vaccines for neglected tropical diseases of humans.

The  $A_{291}Y$  epitope was incorporated into the LCP system, previously shown to stimulate B-cell immune response by activation of dendritic cells via TLR-2 [25,26]. In prior studies, we often observed difficulties in the synthesis and purification of branched LCP constructs and the use of conjugation techniques was necessary [18,27,28,29]. In contrast, herein simple stepwise SPPS was applied for synthesis of all four compounds at high purity. The proper conformation of peptide epitopes is crucial for production of desired B-cell responses, thus we incorporated the  $A_{291}Y$  epitope (AGPKAQVEAIQKY, **1**) in a coil-promoting sequence from the yeast GCN4 protein using a standard technique [15,24] to induce native  $\alpha$ -helical conformation of the  $A_{291}Y$  epitope. Induction of helicity was not significant in the case of chimeric peptide **2**. However, when epitopes **1** and **2** were incorporated into LCP system, both (**LCP-1** and **LCP-2**) adopted more helical than random conformation (Figure 3). This was in agreement with our previous observation in the case of Group A *Streptococcus* vaccine candidates [16,24,28]. Not surprisingly, LCP-1 exhibited  $\alpha$ -helical properties despite this conjugate not containing GCN4 flanking sequences. Induction of  $\alpha$ -helicity of peptides due to their conjugation to dendritic LCP core was previously reported [28]. Similarly, helicity was also induced in peptides conjugated to other dendrimers, presumably due to dense packing of peptide epitopes in such structures [16]. **LCP-1** and **LCP-2** have amphiphilic properties (surfactant-like), therefore there was a risk that such compounds could be toxic to red blood cells. Using a standard haemolytic assay, the capacity of LCP compounds to induce haemolysis was examined and it was clearly demonstrated



**Figure 4. Comparison of antibody responses against peptides with adjuvant vs. LCP-peptides alone.** (a) LCP-2 ( $A_{291}Y-GCN4-LCP$ ) is more immunogenic than peptide 2 ( $A_{291}Y-GCN4$  without LCP) formulated with Freund's adjuvants. Antibody responses are depicted as OD values at a serum dilution of 1:100 where each serum was used to probe its cognate immunogen peptide or LCP. (b) Higher antibody titres were observed in mice immunised with 60  $\mu$ g of LCP-2 without Freund's adjuvants (experiment 2). Total IgG binding to plated LCP-2 from individual mice on day 42 serial dilution from 1:100 to 1:10000. Each symbol represents a single animal. doi:10.1371/journal.pone.0046870.g004

that these compounds were not haemolytic even at high concentration (Figure 2a). The preliminary toxicological studies were performed with Caco-2 cells using an MTT assay (Figure 2b). These human intestinal cells are often used to model toxicity of compounds *in vivo* [30,31,32,33]. No considerable toxicity was observed for any of the tested compounds.

In two separate experiments, LCP-2 induced inconsistent antibody responses in BALB/c mice, characterized by strong

## References

- Hotez PJ, Brooker S, Bethony JM, Bottazzi ME, Loukas A, et al. (2004) Hookworm infection. *New England Journal of Medicine* 351: 799–807.
- Tolentino K, Friedman JF (2007) An update on anemia in less developed countries. *American Journal of Tropical Medicine and Hygiene* 77: 44–51.
- Jones BF, Cappello M (2004) Hookworm infection: molecular mechanisms of disease and targets for control. *Drug Discovery Today: Disease Mechanisms* 1: 217–222.

**Table 1. Neutralisation of the enzymatic activity of recombinant *Na*-APR-1 using purified IgG from mice immunized with LCP-2.**

IgG ( $\mu$ g)	RFU	% inhibition
LCP-2 ms #1 (1.25)	3021	57
LCP-2 ms #1 (2.5)	0	100
LCP-2 ms #4 (1.25)	3463	50
LCP-2 ms #4 (2.5)	312	92
Control IgG (1.25)	6978	–
Control IgG (2.5)	3983	–

Relative fluorescence units (RFU) are corrected to enzyme-free wells which contained substrate alone. Percent inhibition of enzymatic activity with each IgG was determined by establishing the baseline fluorescence using negative control IgG.

doi:10.1371/journal.pone.0046870.t001

antibody responses in some mice and weak responses in others (Figure 4). Similar variability in responses was reported for immunization of mice with an LCP (J8) derived from a Group A *Streptococcus* vaccine candidate [34]. Despite the inconsistency in antibody responses, purified IgG directed against LCP-2 neutralized the enzymatic activity of *Na*-APR-1, the primary goal of this study (Table 1). The ratio of antibody to protease required to neutralize the enzymatic activity was comparable to that obtained with anti- $A_{291}Y$  neutralizing monoclonal antibodies [11].

An ultimate human hookworm vaccine is likely to require at least two antigens, ideally from different developmental stages such as the infective third-stage larva (L3) and the adult worm, to achieve maximum efficacy [7]. *Na*-APR-1 is an ideal candidate antigen from the adult stage parasite, however it is not abundantly expressed in the L3. Antigens secreted by L3, such as *Na*-ASP-2 protect animals against heavy hookworm infections in pre-clinical studies in animals [35], but this antigen elicited an atopic response when administered to people in a hookworm-endemic area due to circulating IgE induced by natural exposure to hookworms [36]. Epitopes were identified in *Na*-ASP-2 that were unique to IgG and not IgE, implying that a peptide-based LCP vaccination strategy focusing on IgG-specific epitopes is a logical approach to developing human helminth vaccines. Such an approach might also integrate multiple epitopes from distinct antigens into the one LCP construct [27,34,37], as well as incorporation of universal T-helper epitopes [38,39,40] to improve consistency in antibody responses and to target outbred human populations. Overall, this work provides the first rational basis for development of the peptide-based vaccine against hookworm infection.

## Author Contributions

Conceived and designed the experiments: MS AL AD MP IT. Performed the experiments: MS AD MK SC. Analyzed the data: MS AD MK MP AL IT. Contributed reagents/materials/analysis tools: AL IT. Wrote the paper: MS. Critically reviewed the manuscript: AD AL IT.

- Albonico M, Smith PG, Ercole E, Hall A, Chwaya HM, et al. (1995) Rate of reinfection with intestinal nematodes after treatment of children with mebendazole or albendazole in a highly endemic area. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 89: 538–541.
- Keiser J, Utzinger J (2008) Efficacy of current drugs against soil-transmitted helminth infections - Systematic review and meta-analysis. *Jama-Journal of the American Medical Association* 299: 1937–1948.

6. Hotez PJ, Bethony JM, Diemert DJ, Pearson M, Loukas A (2010) Developing vaccines to combat hookworm infection and intestinal schistosomiasis. *Nature Reviews Microbiology* 8: 814–826.
7. Loukas A, Bethony J, Brooker S, Hotez P (2006) Hookworm vaccines: past, present, and future. *Lancet Infectious Diseases* 6: 733–741.
8. Pearson MS, Bethony JM, Pickering DA, de Oliveira LM, Jariwala A, et al. (2009) An enzymatically inactivated hemoglobinase from *Necator americanus* induces neutralizing antibodies against multiple hookworm species and protects dogs against heterologous hookworm infection. *FASEB Journal* 23: 3007–3019.
9. Ranjit N, Zhan B, Hamilton B, Stenzel D, Lowther J, et al. (2009) Proteolytic Degradation of Hemoglobin in the Intestine of the Human Hookworm *Necator americanus*. *Journal of Infectious Diseases* 199: 904–912.
10. Loukas A, Bethony JM, Mendez S, Fujiwara RT, Goud GN, et al. (2005) Vaccination with recombinant aspartic hemoglobinase reduces parasite load and blood loss after hookworm infection in dogs. *PLoS Medicine* 2: 1008–1017.
11. Pearson MS, Pickering DA, Tribolet L, Cooper L, Mulvenna J, et al. (2010) Neutralizing Antibodies to the Hookworm Hemoglobinase Na-APR-1: Implications for a Multivalent Vaccine against Hookworm Infection and Schistosomiasis. *Journal of Infectious Diseases* 201: 1561–1569.
12. Skwarczynski M, Toth I (2011) Peptide-Based Subunit Nanovaccines. *Current Drug Delivery* 8: 282–289.
13. Zhong W, Skwarczynski M, Toth I (2009) Lipid Core Peptide System for Gene, Drug, and Vaccine Delivery. *Australian Journal of Chemistry* 62: 956–967.
14. Moyle PM, Toth I (2008) Self-adjuvanting lipopeptide vaccines. *Current Medicinal Chemistry* 15: 506–516.
15. Hayman WA, Brandt ER, Relf WA, Cooper J, Saul A, et al. (1997) Mapping the minimal murine T cell and B cell epitopes within a peptide vaccine candidate from the conserved region of the M protein of group A streptococcus. *International Immunology* 9: 1723–1733.
16. Skwarczynski M, Zaman M, Urbani CN, Lin IC, Jia ZF, et al. (2010) Polyacrylate Dendrimer Nanoparticles: A Self-Adjuvanting Vaccine Delivery System. *Angewandte Chemie-International Edition* 49: 5742–5745.
17. Skwarczynski M, Toth I (2011) Lipid-Core-Peptide System for Self-Adjuvanting Synthetic Vaccine Delivery. In: Mark SS, editor. *Bioconjugation Protocols: Strategies and Methods*, Second Edition. 297–308.
18. Zhong W, Skwarczynski M, Simerska P, Good MF, Toth I (2009) Development of highly pure alpha-helical lipoglycopeptides as self-adjuvanting vaccines. *Tetrahedron* 65: 3459–3464.
19. Zhong W, Skwarczynski M, Fujita Y, Simerska P, Good MF, et al. (2009) Design and Synthesis of Lipopeptide-Carbohydrate Assembled Multivalent Vaccine Candidates Using Native Chemical Ligation. *Australian Journal of Chemistry* 62: 993–999.
20. Gibbons WA, Hughes RA, Charalambous M, Christodoulou M, Szeto A, et al. (1990) Lipidic peptides. I. Synthesis, resolution and structural elucidation of lipidic amino-acids and their homo-oligomers and heterooligomers. *Liebigs Annalen der Chemie*: 1175–1183.
21. Zhong W, Skwarczynski M, Toth I (2009) Development of conformational mimetics of conserved *Streptococcus pyogenes* minimal epitope as vaccine candidates. *Current Drug Delivery* 6: 520–527.
22. Buck M (1998) Trifluoroethanol and colleagues: cosolvents come of age. Recent studies with peptides and proteins. *Quarterly Reviews of Biophysics* 31: 297–355.
23. Bauer MJ, Georgousakis MM, Vu T, Henningham A, Hofmann A, et al. (2012) Evaluation of novel *Streptococcus pyogenes* vaccine candidates incorporating multiple conserved sequences from the C-repeat region of the M-protein. *Vaccine* 30: 2197–2205.
24. Georgousakis MM, Hofmann A, Batzloff MR, McMillan DJ, Sriprakash KS (2009) Structural optimisation of a conformational epitope improves antigenicity when expressed as a recombinant fusion protein. *Vaccine* 27: 6799–6806.
25. Zaman M, Abdel-Aal AM, Phillipps KSM, Fujita Y, Good MF, et al. (2010) Structure-activity relationship of lipopeptide Group A streptococcus (GAS) vaccine candidates on toll-like receptor 2. *Vaccine* 28: 2243–2248.
26. Zaman M, Skwarczynski M, Toth I (2010) Toll-Like Receptor 2 Mediated Dendritic Cell Activation-Key Target for Lipopeptide Vaccines Design. In: Welles LC, editor. *Dendritic Cells: Types, Life Cycles and Biological Functions*: Nova Science Publisher Inc. New York. 63–80.
27. Skwarczynski M, Parhiz BH, Soltani F, Srinivasan S, Kamaruzaman KA, et al. (2012) Lipid Peptide Core Nanoparticles as Multivalent Vaccine Candidates against *Streptococcus pyogenes*. *Australian Journal of Chemistry* 65: 35–39.
28. Skwarczynski M, Ahmad Fuaad AAH, Rustanti L, Ziora ZM, Aqil M, et al. (2011) Group A Streptococcal Vaccine Candidates based on the Conserved Conformational Epitope from M Protein. *Drug Delivery Letters* 1: 2–8.
29. Moyle PM, Olive C, Ho MF, Burgess M, Karpati L, et al. (2006) Method for the synthesis of multi-epitopic *Streptococcus pyogenes* lipopeptide vaccines using native chemical ligation. *Journal of Organic Chemistry* 71: 6846–6850.
30. Lin IC, Liang MT, Liu TY, Ziora ZM, Monteiro MJ, et al. (2011) Interaction of Densely Polymer-Coated Gold Nanoparticles with Epithelial Caco-2 Monolayers. *Biomacromolecules* 12: 1339–1348.
31. Bergeon JA, Ziora ZM, Abdelrahim AS, Pernevi NU, Moss AR, et al. (2010) In Vitro and In Vivo Evaluation of Positively Charged Liposaccharide Derivatives as Oral Absorption Enhancers for the Delivery of Anionic Drugs. *Journal of Pharmaceutical Sciences* 99: 2333–2342.
32. Koeman BA, Zhang Y, Hristovski K, Westerhoff P, Chen YS, et al. (2009) Comparative approach for an in vitro toxicity assay with non-aggregated quantum dots. *Toxicology in Vitro* 23: 955–962.
33. Abid-Essefi S, Bouaziz C, El Golli-Bennour E, Ouannes Z, Bacha H (2009) Comparative Study of Toxic Effects of Zearalenone and Its Two Major Metabolites alpha-Zearalenol and beta-Zearalenol on Cultured Human Caco-2 Cells. *Journal of Biochemical and Molecular Toxicology* 23: 233–243.
34. Olive C, Ho MF, Dyer J, Lincoln D, Barozzi N, et al. (2006) Immunization with a tetraepitopic lipid core peptide vaccine construct induces broadly protective immune responses against group A streptococcus. *Journal of Infectious Diseases* 193: 1666–1676.
35. Bethony J, Loukas A, Smout M, Brooker S, Mendez S, et al. (2005) Antibodies against a secreted protein from hookworm larvae reduce the intensity of hookworm infection in humans and vaccinated laboratory animals. *FASEB Journal* 19: 1743–1745.
36. Diemert DJ, Pinto AG, Freire J, Jariwala A, Hamilton R, et al. (2012) Hookworm vaccination induces urticaria - lessons for the development of human helminth vaccines. *J Allergy Clin Immunol* in press.
37. Moyle PM, Olive C, Ho MF, Good MF, Toth I (2006) Synthesis of a highly pure lipid core peptide based self-adjuvanting triepitopic group A Streptococcal vaccine, and subsequent immunological evaluation. *Journal of Medicinal Chemistry* 49: 6364–6370.
38. Zaman M, Abdel-Aal A-BM, Fujita Y, Phillipps KSM, Batzloff MR, et al. (2012) Immunological Evaluation of Lipopeptide Group A Streptococcus (GAS) Vaccine: Structure-Activity Relationship. *PLoS One* 7: e30146.
39. Abdel-Aal ABM, Zaman M, Fujita Y, Batzloff MR, Good MF, et al. (2010) Design of Three-Component Vaccines against Group A Streptococcal Infections: Importance of Spatial Arrangement of Vaccine Components. *Journal of Medicinal Chemistry* 53: 8041–8046.
40. Abdel-Aal ABM, Batzloff MR, Fujita Y, Barozzi N, Faria A, et al. (2008) Structure-activity relationship of a series of synthetic lipopeptide self-adjuvanting group A streptococcal vaccine candidates. *Journal of Medicinal Chemistry* 51: 167–172.