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Full length article

Target validation of the inosine monophosphate dehydrogenase (IMPDH) gene in *Cryptosporidium* using Phylomer® peptides

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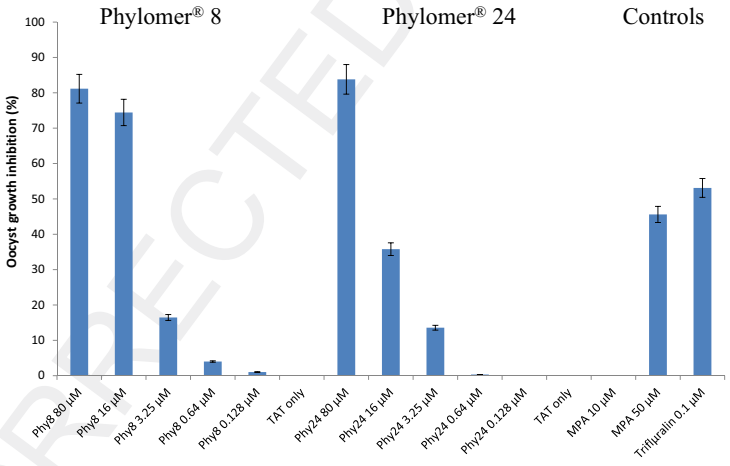
HIGHLIGHTS

- Used novel Phylomer® peptides to validate a drug target in *Cryptosporidium*.
- Yielded a relatively high functional hit rate (~17% i.e. 2/12 Phylomer® peptides tested).
- Successfully shown that TAT can deliver therapeutic Phylomer cargoes inside cells.
- Opens up new therapeutic opportunities.

GRAPHICAL ABSTRACT



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ABSTRACT

Cryptosporidiosis, a gastroenteric disease characterised mainly by diarrheal illnesses in humans and mammals is caused by infection with the protozoan parasite *Cryptosporidium*. Treatment options for cryptosporidiosis are limited, with the current therapeutic nitazoxanide, only partly efficacious in immunocompetent individuals. The parasite lacks *de novo* purine synthesis, and is exclusively dependant on purine salvage from its host. Inhibition of the inosine 5' monophosphate dehydrogenase (IMPDH), a purine salvage enzyme that is essential for DNA synthesis, thereby offers a potential drug target against this parasite. In the present study, a yeast-two-hybrid system was used to identify Phylomer peptides within a library constructed from the genomes of 25 phylogenetically diverse bacteria that targeted the IMPDH of *Cryptosporidium parvum* (IMPcp) and *Cryptosporidium hominis* (IMPch). We identified 38 unique interacting Phylomers, of which, 12 were synthesised and screened against *C. parvum in vitro*. Two Phylomers exhibited significant growth inhibition (81.2–83.8% inhibition; $P < 0.05$), one of which consistently

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exhibited positive interactions with IMPcp and IMPch during primary and recapitulation yeast two-hybrid screening and did not interact with either of the human IMPDH proteins. The present study highlightsthe potential of Phylomer peptides as target validation tools for *Cryptosporidium* and other organisms and diseases because of their ability to bind with high affinity to target proteins and disrupt function.

1. Introduction

Both *Cryptosporidium parvum* and *Cryptosporidium hominis* are important gastrointestinal, waterborne pathogens and the most common non-viral cause of diarrhoea in humans (Xiao, 2010). Cryptosporidiosis can result in severe diarrhoea, dehydration, abdominal pain and weight loss. These symptoms are usually limited to a 2-week duration; however in patients who are immunocompromised, the disease can be persistent and chronic, often becoming life threatening (Carey et al., 2004; Chalmers and Davies, 2010). Currently, nitazoxanide (NTZ) is approved for the treatment of cryptosporidiosis in children and immunocompetent adults in the USA.; however, treatment failures have been reported and NTZ is ineffective for the treatment of immunocompromised individuals (Gargala, 2008; Rossignol, 2010). New therapies are therefore urgently required.

Like other apicomplexan parasites, *Cryptosporidium* is unable to synthesise purine nucleotides de novo and depends solely on the salvage of adenosine from its host or environment (Kirubakaran et al., 2012; Striepen et al., 2004; Umejiego et al., 2004). *Cryptosporidium* does not contain guanine salvage enzymes, and consequently, this pathway appears to be the only route to source guanine nucleotides (Kirubakaran et al., 2012; Striepen et al., 2004). The inosine 5'-monophosphate dehydrogenase (IMPDH) gene appears to have been acquired through lateral gene transfer from a ε-proteobacterium (Striepen et al., 2002, 2004). Detailed kinetic analysis of this prokaryote-like enzyme demonstrated that the *Cryptosporidium* IMPDH is very different from its human homologs (Striepen et al., 2004; Umejiego et al., 2004). Furthermore, the "drugability" of IMPDH is well established as inhibitors of human IMPDHs have been used clinically as immunosuppressants as well as for the treatment of viral infections and cancer (Chen and Pankiewicz, 2007; Hedstrom, 2009; Nair and Shu, 2007). Thus, the exclusive reliance on the salvage pathway by *Cryptosporidium* and its high metabolic demand for nucleotides due to the parasite's complicated lifecycle make IMPDH a potential drug target candidate. This hypothesis is supported by the recent discovery of several *Cryptosporidium* IMPDH inhibitors (Gorla et al., 2012, 2013; Johnson et al., 2013; Maurya et al., 2009; Sharling et al., 2010; Umejiego et al., 2008).

Here we describe a unique integrated target validation platform using IMPDH as an example target. The platform incorporating a Phylomer peptide library (which consists of highly diverse naturally stable protein segments derived from phylogenetically diverse bacterial genomes, that have been optimised for binding to protein surfaces) and interaction of crucial protein interactions using a forward yeast-two hybrid screening assay (Watt, 2006).

Peptides are usually derived from random constrained peptide libraries (peptide aptamers or cyclic peptides) (Colas et al., 1996; Kolonin and Finley, 1998). However, the quality and quantity of hits derived from libraries of randomly encoded sequences are typically low due to the rarity of appropriate structures in such random libraries, even when conformationally constrained (Milech and Watt, 2012; Park and Raines, 2000). In addition, random peptide sequences are more likely to be targeted for degradation making them potentially more unstable than natural peptides (Park and Raines, 2000).

Phylomer peptides represent a very significant advantage over artificial peptides because they are larger than small random peptides and are derived from biological genomes, thereby harnessing the power of natural selection to capture the most stable peptide structures, which have evolved over time (Milech and Watt, 2012). Phylomer libraries are highly enriched for self-scaffolded subdomains (10–50 amino acids long) and protein subdomains (such as those encoded by the Phylomer library) have been shown to fold independently into stable structures (Riechmann and Winter, 2000; Vranken et al., 2002). Phylomers are naturally constrained within their own cognate scaffold structure, rather than artificially constrained within one particular class of fold. Indeed, Phylomer libraries represent more than 1000 classes of protein folds, allowing for considerably more structural diversity. Experiments have demonstrated hit rates for Phylomer peptides, which are capable of disrupting protein interactions, that are around 100-fold better than comparable hit rates obtained using random peptide screening technologies (Blum et al., 2000; Colas et al., 1996; Park and Raines, 2000; Xu et al., 2001). In addition, the potential for reduced side effects is increased because phylomer peptides are derived from bacterial genomes they are unlikely to contain cryptic signalling or binding motifs, which can be recognised by mammalian cells. This significantly reduces the prospect of non-specific interaction and thus toxic side-effects in the host.

The yeast two-hybrid assay is a system for identifying and analysing protein–protein interactions *in vivo*, which has provided insight into many of the protein networks that constitute biological pathways (Coates and Hall, 2003). This assay is based on the modular nature of transcription factors (TF), which can be separated into: (1) the DNA-binding domain (DBD), which mediates binding of the transcription factor to the gene promoter by sequence specific DNA recognition, and (2) the activation domain (AD), which recruits the transcriptional apparatus to the gene for mRNA production. Both activities are required for the induction of gene expression, but they do not have to be present within the same protein (Coates and Hall, 2003; Van Criekinge and Beyaert, 1999). Thus, in a forward two-hybrid assay, interaction between the IMPDH protein fused to a DBD and a Phylomer peptide, fused to an AD, will bring the transcriptional modules into close proximity, reconstituting TF activity and leading to efficient reporter gene induction. However, no gene expression will be seen if the two transcription factor modules do not interact.

Classical forward two-hybrid screening is often used to undertake an "interactor hunt" in which a library of "prey" proteins is screened for interaction against a specific "bait" protein. This provides a method to screen millions of Phylomer candidates to peptides hits that bind specifically to the IMPDH "bait". Once identified, peptide interactors are easily rescued and assessed in downstream assays to identify candidates that can functionally inhibit IMPDH activity. Combined with an established means of delivery of peptides across the *Cryptosporidium* cell membrane using the HIV-1 derived TAT protein transduction domain (PTD) (Chin Lee et al., 2008) and an *in vitro* culture system that supports the entire *Cryptosporidium* life cycle (Hijjawi et al., 2002), this assay provides a useful means of identifying inhibitory peptide-based drug leads in *Cryptosporidium*.

2. Materials and methods

2.1. Forward yeast two-hybrid analysis

In this assay, a dual reporter system was used to identify protein-protein interactions between the IMPDH 'bait' and Phylomer 'prey'. *Saccharomyces cerevisiae* strains SKY 48 (*MATα, his3, tryp1, ura3, 6*

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Table 1
Plasmids used in yeast two-hybrid screening.

Plasmid	Antibiotic	Amino acid	Descriptions	Plasmid size (bp)	Source
pDD	*Kan*	*HIS3*	Bait vector	7309	R. Hopkins
pDD-GilPrep	*Kan*	*HIS3*	Positive control bait		
pSH17-4			Positive control bait		
pSH18-34	*Amp*	*TRY1*	*LacZ* reporter	10,484	P. Watt
pMF45	*Amp*	*URA3*	Prey vector	6483	M. Fear
pJG45-pJGpbx			Positive control prey		

LexA-LEU2, lys2::3 clop-LYS2) transformed with the 'prey' plasmid (pDD) containing the activation domain, and SKY 473 (*MATa, his3, tryp1, ura3, 4 LexA-LEU2, lys2::3 clop-LYS2, CAN*^{R}) transformed with pSH18-34 (LacZ, URA3) and the 'bait' plasmid (pMF45) which contained the DNA binding domain, were used for yeast two-hybrid assays. Further details of the plasmids used are provided in Table 1. We postulated that if the 'bait' protein (IMPDH) interacted with the Phylomer 'prey' peptide, transcription of various reporter genes (e.g. LEU2 and LacZ) would be initiated. Reporter gene activity could then be monitored using a standard X-gal colorimetric (blue/white) and auxotrophic selection assays. Blue colonies containing the interacting peptide could then be 'rescued' and the interacting peptide identified. In the absence of any bait/prey interaction, transcription would not occur and the reporter gene would not be switched on leaving the colonies white in the presence of an X-gal substrate.

2.1.1. Phylomer peptide library construction

The Phylomer library was generated by amplifying short DNA fragments from the genomes of 25 unique bacterial species (American Type Culture Collection, ATCC) as described in Milech and Watt (2012). Products from the whole-genome PCR amplification procedure were cloned into pMF45 Gateway (AX STOP) plasmid vector (Invitrogen, Carlsbad, CA, USA) and the recombinants transformed into *S. cerevisiae* strain SKY 48 yielding a Phylomer library complexity of 1.34×10^8 cfu/mL independent clones.

2.1.2. IMPDH bait plasmid construction

DNA was extracted from cultured *C. parvum* (IOWA strain) and *C. hominis* (H153) as previously described (Hijjawi et al., 2010). The entire open reading frames (ORFs) of the IMPDH gene for both species were PCR amplified with Pfu Ultra fusion II (Stratagene, La Jolla, CA, USA) using the primers IMPc F2 (5' AA**GAATTC**ATGGGTA CAAAAAACATAGGAAAAG 3') and IMPc R2 (5' AA**GCGGCCGC**CTAT TTACTATAATTCATTACTTCTTTTACG 3'). *Eco*RI and *Not*I restriction sites (shown respectively in bold and underlined) were used to clone the PCR fragments into the pDD vector to form the plasmids pDDIMPcp and pDDIMPch. Similarly, the entire ORF for human IMPDH1 and IMPDH2 were also PCR amplified from lung cDNA (Ambion, Austin, TX, USA) using the primers IMP1 F2 (5' AA**GCGGCCGC**ATGGAGG GGCCACTCACTCCACCACC 3') and R2 (5' AA**CTCGAG**TCAGTACAGC CGCTTTTCGTAAGAGTGC 3') for IMPDH1 and IMP2 F2 (5' AA **GCGGCCGC**ATGGCCGACTACCTGATTAGTGGGGGCACG 3') and R2 for IMPDH2 (5' AA**CTCGAG**TCAGAAAAGCCGCTTCTCATACGAATGG 3'). The restriction sites *Not*I and *Xho*I were used to clone the PCR fragment into the pDD plasmid to form the specificity controls, pDDIMPh1 and pDDIMPh2. Both the amplified PCR products and the constructed bait plasmid were sequenced to ensure no mutational errors were incorporated into the target gene sequence and that all constructs contained in-frame coding sequences.

2.1.3. IMPDH expression in yeast

Each of the pDD-derived plasmids were transformed into SKY 48 yeast strains and expression of the 'bait' as LexA-fusion proteins was induced by the presence of 2% galactose (and 2% raffinose to sustain growth) in selective growth media. Protein was extracted from yeast using 50 mM TRIS, pH 7.5; 10 mM sodium azide on ice, then resuspended in electrophoresis sample buffer and run on a 10% SDS-page gel. After Western transfer to a nitrocellulose membrane (Hybond, Escondido, CA, USA), target proteins were identified by incubating the membrane with the primary antibody, 1:1000 anti-LexA mouse monoclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% Skim Milk Powder/Western Wash buffer for 1 h. The membrane was rinsed three times in Western wash buffer and then washed three times for 5 min in Western wash buffer with shaking. After washing, a secondary antibody, 1:5000 anti-mouse horseradish peroxidase (Amersham, Piscataway, NJ, USA) in 15 mL Western wash buffer was added for 1 h. After three rinses and two 5 min washes in Western wash buffer with shaking, the antibody binding was visualised using an enhanced chemiluminescence (ECL) antibody detection kit (Amersham) and autoradiograph exposure (AGFA CP1000, Düsseldorf, Germany).

2.1.4. Testing for auto-activation

To test for *LEU2* and *LacZ* auto-activation, an initial screen was conducted using SKY 473 transformed with the test bait plasmids (pDDIMPcp, pDDIMPch, pDDIMP1 and pDDIMP2). Positive control bait plasmids (pDD-GilPrep and pSH17-4) were plated onto UHWL$^-$ plates with 2% raffinose and a range of galactose (0.1–2%) concentrations. SKY 48 was transformed with the positive control prey plasmid (pJG45-pJGpbx) and plated onto UHW$^-$/X-Gal overlay assay plates with 2% raffinose and a range of galactose (0.1–2%) concentrations. Media plates with 2% glucose and 2% raffinose media were used as the negative controls.

2.1.5. Yeast mating

Small-scale screens of the Phylomer library were then conducted independently using IMPDH genes derived from of *C. parvum* (pDDIMPcp) and *C. hominis* (pDDIMPch) respectively. 1×10^8 cfu/mL of SKY473/pDDIMPcp/pSH18-34 yeast were mated with 1×10^7 cfu/mL of the SKY48/Phylomer library and 2.5×10^7 cfu/mL of SKY473/pDDIMPch/pSH18-34 yeast were mated with 2.5×10^6 cfu/mL of the SKY48/Phylomer library. A minimum of 2.5×10^7 cfu/mL of SKY473 yeast containing either pDDIMPcp/pSH18-34 or pDDIMPch/pSH18-34 plasmids were mated with SKY48 yeast containing the Phylomer library at a ratio of 10:1. Matings were plated onto UHW- plates with 0.1% galactose (optimal concentration from *LacZ* auto-activation testing) to select for diploids. Yeast colonies were harvested and resuspended in yeast freezing buffer and 200 μL aliquots were frozen at –80 C.

2.1.6. Selection of interactors

Diploids were plated onto UHWL-X media as a dual selection for yeast that were positive for LEU (growth) and Lac Z expression (blue colouration). To reconfirm true positive interactions, the galactose-dependent transcriptional activation of both *LEU2* and *LacZ* reporters of putative interactors were assessed. All positive colonies (blue), along with three white colonies from each library mating (as negative controls), were selected from UHWL$^-$X plates before being spotted onto UHWL$^-$ and UHW$^-$ (followed by X-Gal overlay assay)

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media plates. Raffinose (2%) with 1% and 0.1% galactose were included to compare the expression of the interactors. Media plates with 2% glucose and 2% raffinose media were used as the negative controls.

2.1.7. Characterisation of positive interactors

To characterise the Phylomers that exhibited interactions with IMPch and IMPcp, the extracted plasmids were PCR amplified using the following mixture: 2 µL of 2 mM dNTP mix (Fisher-Biotech, Perth, WA, Australia), 0.4 µL of *DyNAzyme EXT* (Finnzymes Oy, Denmark), 2 µL of 10 × *DyNAzyme EXT* Reaction Buffer (Finnzymes Oy), 4 µL of each 1 µg/µL BC01 (5' CCAGCCTCTTGCTGAGTGGAGATG 3') and BCO2 (5' CTGTTCGGCTGTTGGAACTAACCTC 3') (Geneworks, Thebarton, SA, Australia). PCR reactions containing 2 µL of extracted plasmid were amplified in a total 20 µL reaction volume. Reactions were cycled using a PCR machine PTC-200 Peltier Thermal Cycler using the following cycles: an initial melt at 94 °C for 10 min, then 30 cycles of melting at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min 30 s, with a final 10 min extension time. pMF 45 Gateway, pDD and pSH 17-4 were used as negative controls.

PCR products were sequenced with BC01 and BC02 primers. Sequences were analysed using 4peaks (version 1.7, Apple Computer, Inc) and MacVector (version 7.1, Oxford Molecular Group), and the forward and reverse products were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/) to ensure the absence of sequencing error. Nucleotide sequences were then compared with sequence data available from GenBank™ using the BLAST 2.2.9 program (http://www.ncbi.nlm.nih.gov:80/BLAST/). Protein sequences were translated and assessed for ORFs using ORF Finder (http://www.ncbi.nlm.gov/projects/gorf/) before being aligned using ClustalW.

2.1.8. Recapitulation of interacting Phylomers

Library plasmids were rescued from diploid yeast and then transformed into TOP 10 *E. coli* grown on AMP selection media. Colonies were then screened using PCR to identify cells positive for pMF45 plasmids rather than pSH18-34 (also AMP resistant). Plasmids were purified from the positive colonies and then transformed back into haploid SKY 48 yeast. Mating was then conducted between the SKY 48 yeast containing the positive Phylomers and SKY 473 yeast containing pDDIMPch, pDDIMPcp or the specificity control targets pDDIMP1 and pDDIMP2 to assess recapitulation of the primary screen results and to determine levels of cross-reactivity of the Phylomers with the human IMPDH proteins. Colonies were plated onto $UHWL^-$ and UHW^- (followed by X-Gal overlay assay) media to assess growth and LacZ expression.

2.2. Phylomer synthesis for in vitro screening

Phylomers were custom synthesised as TAT-fusion proteins by Mimotopes Pty Ltd (Notting Hill, VIC, Australia) and dissolved in 100 µL of 80% DMSO in water to obtain a primary stock concentration of 8 mM. Phylomer-DMSO solution was incubated at room temperature for 30 min, then vortexed to encourage dissolving. Aliquots were stored at –20 °C.

2.3. C. parvum oocyst purification and excystation

C. parvum cattle isolate (SC26), originally obtained from the Institute of Parasitology, University of Zurich was purified using ether extraction and a ficoll density gradient as previously described (Meloni and Thompson, 1996). Purified oocysts were stored in sterile 1× phosphate buffered saline (1× PBS) and an oocyst count was performed on a haemocytometer before storing at 4 °C.

Oocysts were further bleached and excysted as previously described (Boxell et al., 2008), then resuspended in 25 mL of maintenance media filter sterilised via a 0.22 µm vacuum filter and stored at 37 °C ready for host cell infection. Maintenance medium consisted of RPMI 1640 (SIGMA-ALDRICH®) medium (10.3 g/L) supplemented with L-glutamine (0.3 g/L), sodium bicarbonate (2 g/L), glucose (1 g/L), bovine bile (0.2 g/L), 15 mM HEPES buffer (3.574 g/L), folic acid (0.25 g/L), 4-aminobenzoic acid (1 g/L), calcium pantothenate (0.5 g/L), ascorbic acid (8.75 g/L) and 1% foetal calf serum (FCS) and the pH adjusted to 7.2. No antibiotics were added to minimise possible effects on the Phylomer screening assays.

2.4. Cell culture

Excysted *C. parvum* oocysts were cultured in human ileocecal adenocarcinoma cell line 8 (HCT-8) host cells (ATCC 244). These cells were cultured to monolayer in growth media consisting of RPMI 1640 (SIGMA-ALDRICH®) medium (10.3 g/L) supplemented with L-glutamine (0.3 g/L), sodium bicarbonate (2 g/L), 15 mM HEPES buffer (3.574 g/L) and 10% FCS (pH 7.2). Media was filter sterilised using a 0.22 µm vacuum filter and no antibiotics were added to the media. Media sterility tests were performed with each passage of cells and screen setup. Cells were maintained in 72 cm^2 flasks with filter tops (CELLSTAR®) in 5% CO_2 at 37 °C as previously described by Hijjawi et al. (2001). Phylomer screens were set up by removing cell monolayers from culture flasks using 1 mL of trypsin/EDTA (0.05% (w/v) trypsin; 0.53 mM EDTA) and inoculating into 48 well plates (CELLSTAR®).

2.5. In vitro testing of Phylomers

The growth medium from each 48 well plate was discarded and the cell monolayers were washed once in sterile 1× PBS. The 70–80% confluent HCT-8 monolayers of cells were infected via placing 495 µL aliquots of the excysted *C. parvum* oocysts (approximately 16,000 oocysts/mL) in maintenance media (approximately 8000 oocysts/well). As a control, intact but heat inactivated oocysts (32,000 oocysts/mL incubated at 70 °C for 30 min) were also processed for each assay. Aliquots of 495 µL of oocysts in maintenance media were placed into four control wells and incubated for 24 h in 5% CO_2 at 37 °C.

Phylomers were introduced into cultures at 24 h post infection at concentrations of 80, 16, 3.2, 0.64 and 0.128 µM. Positive controls were HCT-8 cells infected with oocysts, while negative controls were HCT-8 cells with heat killed oocysts and uninfected HCT-8 cells. Five microlitres of 80% DMSO was added to positive controls to ensure that DMSO at < 1% did not inhibit HCT-8 cell growth. Duplicate TAT only peptide controls were included in each screen at a concentration of 80 µM. Each Phylomer was tested in vitro at least eight times.

Each screening plate also included two positive drug controls; trifluralin (TF) (0.1 µM) which has previously been shown to produce 50–70% inhibition of *C. parvum* growth *in vitro* at that concentration (Armstrong, unpublished data) and mycophenolic acid (MPA), which is a weak inhibitor of IMPDH (10 and 50 µM) (Umejiego et al., 2004). Screen plates were incubated for 48 h in 5% CO_2 at 37 °C.

2.6. DNA extraction and quantitative PCR (q-PCR)

Prior to DNA extraction, all cell monolayers were observed for any disruption and wells that contained a detached monolayer of cells or a high number of dead cells were not processed for the quantitative PCR assay (qPCR). Detached and dead cells indicate unhealthy cell monolayer, which would have hindered the growth of *C. parvum in vitro* (Hijjawi et al., 2001; Upton et al., 1995). Maintenance medium was removed from wells by aspiration and discarded. The cell monolayers were washed once in sterile 1× PBS. To aid detachment of the cells from the well surface, 70 µL of 0.5% Trypsin /EDTA was added

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to each well and incubated for 24 h in 5% CO_2 at 37 °C. Monolayers were harvested using 1× PBS and centrifuged for 10 min at 20,800 × *g*. The supernatant was removed via aspiration after which, 20 µL of AE buffer (Qiagen, Hilden, Germany) was added and vortexed to resuspend the pellet. The samples were subjected to five freeze (liquid nitrogen)-thaw (100 °C) cycles (~30 s each), following which they were boiled at 100 °C for 20 min. Samples were left to cool, centrifuged at 10,000 × *g* for 3 min and stored at –20 °C before being used for the qPCR assay.

qPCR was performed in triplicate using a *Cryptosporidium* diagnostic locus unique to *Cryptosporidium* as previously described (Yang et al., 2009). A standard curve was constructed using five triplicates of genomic DNA extracted from known numbers of oocysts and serially diluted at a 1:9 ratio, calibrated to correspond to 1–10,000 oocysts. Statistical analysis was performed using SPSS 17.0 (Statistical Package for the Social Sciences) for Macintosh OS X (SPSS Inc., Chicago, IL, USA) to determine if there was a statistically significant decrease in parasite DNA in Phylomer treated wells compared with no Phylomer treated controls.

2.7. DNA standard preparation and data analysis

Oocyst DNA (15,000 oocyst/µL) was serially diluted 10-fold in 1× PCR buffer II (Genworks). DNA extracted from samples containing between 15,000 and 1.5 oocyst/µL was used to generate a standard curve for each screen run using Rotor-Gene 6.0.14 software. Percentage (%) inhibition of *C. parvum* growth was calculated using average DNA concentrations of the positive control and test assays. DNA concentration was analysed by a one-way ANOVA (SPSS version 17.0) and transformed by square root and log to stabilise variances between Phylomer introduced groups. A LSD test to compare the effect of each Phylomer with the positive control was also conducted (SPSS 17.0).

2.8. Guanine recovery assay

As IMPDH targets guanine salvage, a guanine recovery assay was performed in the presence of Phylomer 24 (as this was the only Phylomer® that interacted with *Cryptosporidium* IMPDH and not human IMPDH), to determine if addition of guanine resulted in reversal of inhibition. Cultures were incubated with Phylomer 24 at 16, 3.25, 0.64 and 0.128 µM, respectively for 48 h and then 50 µM guanine was added to each well, a further 200 µL cell growth media was added and the plates were incubated at 37 °C for another 48 h. At this point, the wells were washed and the DNAs were extracted, followed by qPCR.

2.9. Measurement of cytotoxicity of Phylomers

Cytotoxicity to host cells was determined using the TOX7 kit (Sigma-Aldrich), which measures lactate dehydrogenase (LDH) as a guide to cell integrity. The assay was performed according to the manufacturer's instructions and both positive and negative controls were used. The assay was performed in quadruplicate.

3. Results

The entire ORF for the IMPDH gene of *C. parvum* and *C. hominis* was successfully PCR-amplified and cloned into a yeast expression as a C-terminal fusion with the LexA protein (Fig. 1A). Expression of pDDIMPch, and pDDIMPcp-Lex A fusions in yeast yielded proteins of the expected size of 67 kDa. Expression of vector-only pDD controls, containing only the LexA protein generated 22 kDa proteins of the expected size (Fig. 1B). pDDIMph1 and pDDIMPh2 expressed as larger fusion proteins of 81 and 77 kDa, respectively.

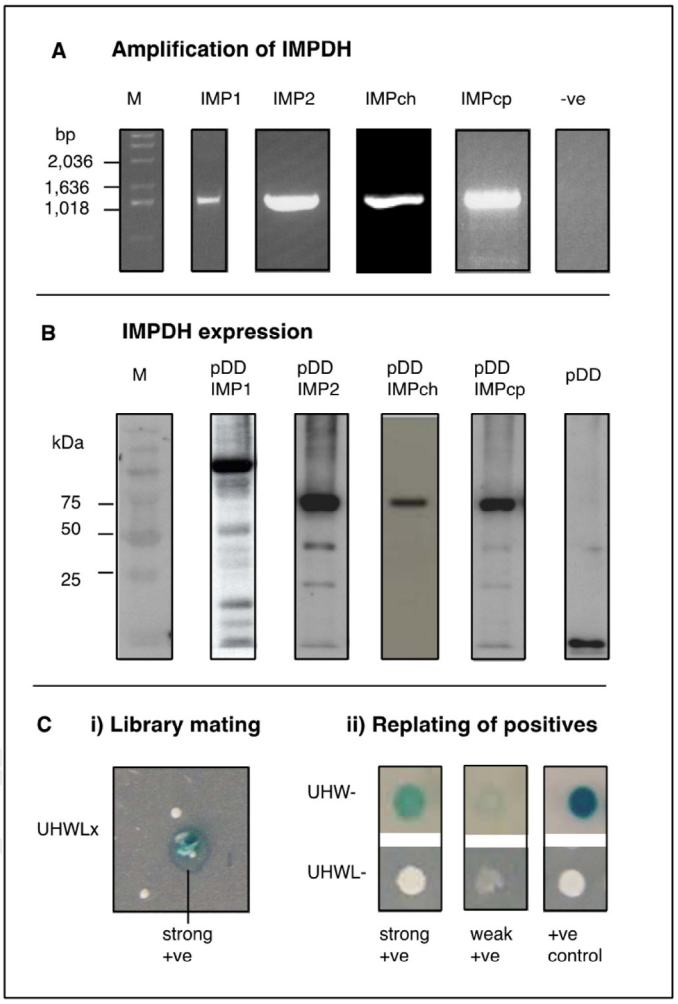

Fig. 1. Plasmid construction and expression. (A) Ethidium bromide stained 1% agarose gel electrophoresis of PCR amplified IMPDH genes. M: 1kB DNA Ladder; IMP1: human IMPDH1 (1837 bp); IMP2: human IMPDH2 (1582 bp); IMPcp: *C. parvum* IMPDH gene (1217 bp), IMPch: *C. hominis* (1217 bp); Negative control. (B) Western blot analysis of LexA-fusion 'bait' protein expression in yeast for pDDIMP1(~77 kDa), pDDIMP2, pDDIMPcp and pDDIMPch (67 kDa). M: Protein marker. Empty pDD vector shows expression of LexA (25 kDa). (C) Example of a positive interactor from an initial library screen on a UHWL-X plate (i) and examples of replated interactors on UHW- and UHWL- plates (ii).

DNA sequencing revealed 100% and 99% homology respectively to *C. parvum* IMPDH (GenBank accession no: XM_625342 [*C. parvum*] and HM071002 [*C. hominis*]). Four amino acid differences were observed between the translated sequences of *C. parvum* and *C. hominis*. Sequences have been submitted to GenBank under the accession numbers KF049205 and KF049204.

3.1. Yeast forward-two hybrid analysis

LEU2 and *LacZ* auto-activation were not observed in yeast (SKY473) expressing only full length bait proteins (pDDIMPch, IMPcp, IMP1 and IMP2) as evident from the lack of growth on $UHWL^-$ plates at various galactose concentrations. In contrast, the positive controls pSH17-4, GilPrep1/pJGpbx and GilPrep2/pJGpbx clones showed effective growth (data not shown).

Of 5×10^8 pDDIMPch diploids, 25 million were screened on UHWL- + x-gal media. Forty four colonies exhibited activation of both reporter genes and were therefore considered potential interactors. Of 2×10^9 pDDIMPcp diploids, 15 million were screened on $UHWL^-$ + x-gal media and 29 colonies exhibited expression of both

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reporter genes. Average mating efficiency was calculated to be 49% and 73% for the pDDIMPch and pDDIMPcp screens, respectively. Positive clones were then grown on separate UHW- and UHWL- plates and those that exhibited the highest levels of growth and b-gal expression (positive for LEU- and X-gal) were sequenced ($n = 37$).

3.2. Sequence analysis of putative interactors

Nucleotide and amino acid sequences for putative interacting Phylomer peptides ($n = 37$) were searched against the GenBank™ database using BLASTn and BLASTp respectively. Seven peptides were identified more than once in each screen and two were further identified independently in both the pDDIMPcp and pDDIMPch screens. All nucleotide sequences for each peptide matched a known bacterial species within the original library and four peptide sequences were homologous to natural ORFs (a subselection of Phylomers are shown in Table 2).

Amino acid sequences were further aligned and while few amino acids were identical or similar among the interactors, proline and lysine were moderately conserved in most of the interacting Phylomer peptides (data not shown).

3.3. Recapitulation of interacting Phylomers

Of the 38 unique Phylomers identified in the primary screens, 30 were rescued and then tested in a recapitulation screen to confirm interaction with the original target and also to determine the specificity of interaction against IMPcp and IMPch, and also the related human proteins IMPDH1 and IMPDH2. All screens were conducted in duplicate on UHW- and UHWL- plates. A total of six Phylomers were found to interact strongly with both human IMPDH proteins, and 12 and three Phylomers also exhibited positive interactions with either IMPDH1 or IMPDH2, respectively. Results of these interactions for a subset of phylomers tested in vitro are shown in Table 2. TAT-fusion Phylomers ($n = 12$) and a TAT-only control (Table 1) were subsequently commercially synthesised for *in vitro* analysis (Table 2).

3.4. In vitro screening of Phylomers

Microscopic (40×) and visual observations made prior to *C. parvum* infection, showed that HCT-8 cell monolayers were undisturbed in each well and had grown to a confluence of approximately 70–80%. Post infection and prior to adding Phylomers, there were large numbers of *C. parvum* oocysts and stages visible in each culture well, except in the two negative control wells as expected.

Some Phylomers precipitated when added to media and therefore it was decided to redissolve all Phylomers in 10% acetonitrile and retest. When testing Phylomers dissolved in acetonitrile, an acetonitrile control (at 0.1% or 0.5 μM, which was equal to the concentration of acetonitrile in the 80 μM Phylomer treatment) was included in the screen plate. Acetonitrile-only controls had no effect on *C. parvum* growth in vitro (data not shown). The effect of Phylomers on parasite growth was quantified via microscopy and via qPCR analysis of total DNA extracted from each well as described in Section 2.7.

Of the Phylomers tested, two peptides (Phylomers 8 and 24) were found to significantly inhibit *C. parvum* growth in vitro in a dose–response manner (Fig. 2). Phylomer 8 reduced *C parvum* growth by 81.2%, 74.4%, 16.5%, 4.0% and 1.0% after incubation with 80, 16, 6.4, 3.25, 0.64 and 0.128 μM of peptide respectively. The IC_{50} was 8 μM. Wells containing TAT-fused Phylomer 8 peptide exhibited significant differences in growth inhibition compared with the TAT-only control peptide regardless of concentration ($F = 177.00$; $F_{Critical} = 3.11$; $P < 0.05$). There was also a significant difference between the higher concentration Phylomers (80 and 16 μM) and the lower concentration Phylomers (3.25, 0.64, 0.128 μM) ($P < 0.05$). There was no significant difference between the 80 and 16 μM treatments.

Addition of Phylomer 24, also produced a dose–responsive inhibition of *C. parvum* growth *in vitro* with inhibition of 83.8%, 35.8%, 13.6%, 0.3% and 0% inhibition respectively at the five Phylomer concentrations tested and an IC_{50} of 46 μM. There was a significant difference among the five Phylomer concentrations and the no Phylomer TAT only control ($F = 177.00$; $F_{Critical} = 3.11$; $P < 0.05$) and there was also a significant difference between 80 and 16 μM treatments. There was a linear correlation between Phylomer 24 concentrations added and *C. parvum* inhibition ($R^2 = 0.97$). At 80 μM neither Phylomer caused a detectable increase in lactate dehydrogenase concentrations in the cell media using the TOX7 kit (Sigma-Aldrich).

The control drug MPA inhibited parasite growth by 45.6% at 50 μM ($P < 0.0053$). Trifluralin inhibited parasite growth by 53.1% at 0.1 μM ($P < 0.001$).

3.5. Guanine recovery assay

An inverse relationship was observed between Phylomer 24 concentrations and guanine recovery. For example, the lowest

Table 2
Phylomers® tested *in vitro* against *Cryptosporidium*.

Phylomer® number	Initial library interaction	Recapitulation interactions		Peptide sequence	Organism (BLASTn)
		Cryptosporidium IMPDH	Human IMPDH		
2 (chS1X1)	IMPch		IMPDH1	H-**GRKKRRQRRRG**PSHPTKKHLNHSKRANFKT-NH_2	*Listeria innocua*
4 (chS1X4)	IMPch		IMPDH1	H-**GRKKRRQRRRG**LQSWRCLQDV-NH_2	*Aeropyrum pernix*
6 (chS1X10 + chS1L12)	IMPch	IMPcp,	IMPDH1	H-**GRKKRRQRRRG**PPLISRLIKKKQI-NH_2	*Shigella flexneri*
8 chS2A1	IMPch	IMPcp,	IMPDH1	H-**GRKKRRQRRRG**LAQIRSFPFSVNIFLRKSVFQST-NH_2	*Haemophilus influenzae*
10 chS2B1 cpS1B1 cpS2B2 cpS2B4	IMPch; IMPcp		IMPDH1, IMPDH2	H-**GRKKRRQRRRG**LGFHFHQMSFLLRY-NH_2	*Escherichia coli*
12 chS2C3	IMPch			H-**GRKKRRQRRRG**PSLRKS-NH_2	*Listeria innocua*
14chS2D1	IMPch	IMPcp,	IMPDH1	H-**GRKKRRQRRRG**PSSVPASMRVP-NH_2	*Bordetella pertussis*
16 chS2H1	IMPch	IMPcp		H-**GRKKRRQRRRG**LRWCCKSHHKPIPQANCCFLMSR-NH_2	*Shigella flexneri*
18 cpS2B3	IMPcp		IMPDH1, IMPDH2	H-**GRKKRRQRRRG**QTHRTGRSPSPTRFA-NH_2	*Salmonella enterica*
20 cpS2B7	IMPcp			H-**GRKKRRQRRRG**PRSHQPYRLFHLERP-NH_2	*Listeria innocua*
22 cpS2B6	IMPcp		IMPDH1	H-**GRKKRRQRRRG**LKTIITNQS-NH_2	*Staphylococcus aureus*
24 chS2G1 cpS1G1	IMPch; IMPcp	IMPcp, IMPch		H-**GRKKRRQRRRG**LPGPNIYLFFFVRSMTCLDLRPLTRPCSPR-NH_2	*Chlorobium tepidum*
26	-			H-**GRKKRRQRRRG**-NH_2	TAT only control

IMPcp = *C. parvum* IMPDH; IMPch = *C. hominis* IMPDH.

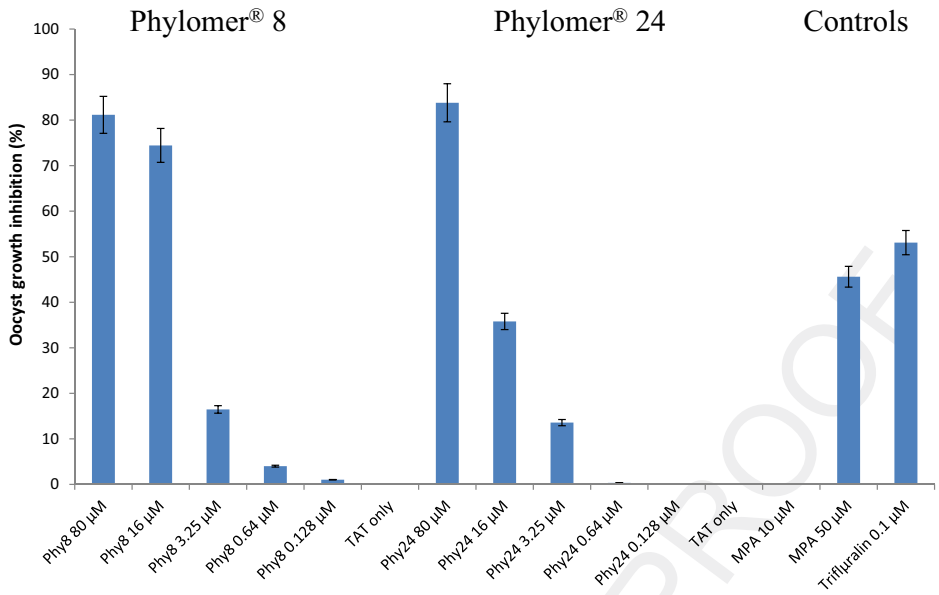

Fig. 2. Dose–response effect of Phylomers® 8 and 24 on *C. parvum* growth inhibition *in vitro*. The error bars represent the 95% confidence intervals of parasite growth reduction. The concentration range of Phylomers® 8 and 24 was 80, 16, 3.2, 0.64 and 0.128 µM. MPA was tested at 10 and 50 µM. TF was tested at 0.1 µM. The TAT only control was tested at 80 µM.

concentration of Phylomer 24 tested (0.128 µM) resulted in complete recovery following the addition of 50 µM guanine (100%) (Table 3) The highest Phylomer concentration tested (16 µM) resulted in 20% recovery. There was a significant difference ($F = 0.151$; $F_{Critical} = 0.108$) between the different Phylomer concentrations tested. The no guanine treated controls and the TAT-only controls exhibited no decrease in inhibition.

3.6. Cytotoxicity assays

Cytotoxicity assays revealed that both Phylomer 8 and 24 exhibited negligible cytotoxicity levels on the HCT-8 cells (0.08% and 0.07% respectively for Phylomer 8 and 24 at the highest concentration tested (80 µM). MPA exhibited 2.9% and 2.3% cytotoxicity at the 50 and 10 µM concentrations tested. 0.1 µM. TF exhibited 2.3% toxicity and the TAT only control exhibited no cytotoxic effects.

4. Discussion

Cryptosporidium is an enteric parasite, which has a global impact on the health, survival and economic development of >10 million people and animals world-wide (Xiao, 2010). Despite this, efforts to develop new and more effective treatments for this disease have been hindered by (1) lack of suitable targets and (2) lack of target validation and transfection tools. The completion of the *Cryptosporidium* genome (Abrahamsen et al., 2004; Xu et al., 2004), which resulted in a range of potential drug targets being identified, has overcome the first hindrance and IMPDH is now a well recognised and important drug target (Gorla et al., 2012, 2013; Johnson et al., 2013; Maurya et al., 2009; Sharling et al., 2010; Umejiego et al., 2008).

Our study successfully developed an integrated technology platform that facilitates high throughput validation of drug targets in *Cryptosporidium* using IMPDH as an example. This system incorporates a genetic-based screening module which utilises high throughput yeast two-hybrid technology in conjunction with a unique peptide library, highly enriched for structures that have evolved to bind to protein interfaces, to screen for high affinity Phylomer peptide-IMPDH interactions. Peptides rescued from these screens were then tested for their ability to inhibit *Cryptosporidium* growth *in vitro* as assessed by a qPCR assay.

In order to test the anti-cryptosporidial activity of Phylomers, it was necessary to transport the Phylomers across the *Cryptosporidium* cell membrane. While cell membranes are normally impermeable to peptides, the recent discovery of short cationic peptides that cross the plasma membrane efficiently has opened up new possibilities for the intracellular delivery of such agents. These peptides are commonly referred to as protein transduction domains (PTDs) and have been successfully used to transport heterologous proteins, peptides and other types of cargo into cells (Kabouridis, 2003). The TAT protein, a transactivation factor from the human immunodeficiency virus 1 (HIV-1), was the first polypeptide shown to gain entry into cells when added exogenously to culture medium (Green and Loewenstein, 1988). The capability of TAT conjugated peptides as a delivery vehicle into *Cryptosporidium* and other protozoan parasites has previously been demonstrated using TAT fused with Green Fluorescent Protein (TAT-GFP) (Chin Lee et al., 2008). In that study, 100% of *C. parvum* life-cycle stages including oocysts fluoresced when native TAT-GFP was introduced into *C. parvum* cultures (Chin Lee et al., 2008). This provides for the first time a means to transfect target validation reagents into the parasite and represents a significant advance in the development of target validation tools for *Cryptosporidium*.

Of the 12 synthetic Phylomers screened, two (Phylomer 8 and 24) exhibited significant anti-cryptosporidial activity in vitro. Phylomer 8 is a 23 amino acid peptide derived from *Haemophilus influenzae* and Phylomer 24 is a 32 amino acid peptide derived from

Table 3
Guanine recovery efficiency assay tested on Phylomer 24.

Phylomer concentrations	No guanine inhibition rate (%)	Inhibition rate (%) after addition of 50 µM guanine	Recovery rate (%)
TAT only control	0	0	0
0.128 µM	6.0	0	100
0.64 µM	29.4	13.2	55
3.25 µM	51.0	35.0	31
16 µM	80.0	64.0	20

Chlorobium tepidum. Both Phylomers exhibited 81.2–83.8% inhibition respectively of *Cryptosporidium in vitro* and IC_{50}s of 8 and 46 μM. Furthermore, Phylomer 24 consistently exhibited positive interactions in the primary and recapitulation yeast two-hybrid screens and did not interact with either of the human IMPDH proteins. These findings further support the therapeutic potential of targeting the *Cryptosporidium* IMPDH via the identification of prospective interacting peptides that also exhibit anti-cryposporidial activity *in vitro*.

Our study focused on proving the usefulness of the Phylomer peptides for target validation in *Cryptosporidium*, an organism that has traditionally been proven intractable to such high throughput screening approaches. However in recognition of the fact that peptide-based anti-parasitic drugs are unlikely to be either affordable or commercially viable in developing-world settings, future studies will focus on utilising bioinformatics to identify a range of potential targets, which we can be subsequently validated as therapeutic targets using our novel system. Small molecular compounds to disrupt these targets will be designed and assayed for anti-*Cryptosporidium* activity *in vitro* and *in vivo*.

In conclusion, the novel Phylomer approach described here has application in a wide range of organisms and disease states and can be used to reduce/expedite the process of identifying suitable drug targets for a range of pathogens and diseases. Phylomers have enormous potential as target validation tools for *Cryptosporidium* and other organisms and diseases because they are able to bind with high affinity to target proteins and disrupt function. The screening approach used in the present study yielded a relatively high functional hit rate (~17% i.e. 2/12 Phylomer peptides tested). We also successfully validated the concept that cell penetrating motifs such as TAT can be used to deliver therapeutic Phylomer (biologics) cargoes inside cells where they can act upon the parasites which opens up new therapeutic opportunities. Phylomers can also be used not just be as biological probes to validate 'druggable' targets but also to map binding interfaces to identify therapeutically relevant epitopes that can be used to direct the development alternative more cost effective therapies (e.g. small molecules). This approach will also assist in elucidating our understanding of the basic biology of *Cryptosporidium* and other organisms by providing a mechanism to surgically knock-out proteins involved in a variety of cellular processes and identify how different proteins are required for interaction with each other.

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