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Phage display identification of functional binding peptides against 4-acetamidophenol (Paracetamol): An exemplified approach to target low molecular weight organic molecules

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Abstract

Peptide-phage display has been widely used to explore protein–protein interactions, however, despite the potential range of applications the use of this technology to identify peptides that bind low molecular weight organic molecules has not been explored. In this current study, we identified a phage clone (PARA-061) displaying the cyclic 7-mer peptide sequence $^{N'}$AC-**NPNNLSH**-CGGGS$^{C'}$ that binds the low molecular weight organic molecule 4-acetamidophenol (4-AAP; paracetamol). To avoid occupancy of key functional groups on the target 4-AAP molecule our panning strategy was directed against insoluble complexes of 4-AAP rather than against the target linked to a stationary support or bearing an affinity tag. To augment the panning procedure we deleted phage that also bound the 4-AAP isomers, 2-AAP and 3-AAP. The identified PARA-061 peptide-phage clone displayed functional binding properties against 4-AAP in solution, able in a peptide sequence-dependant manner to prevent the *in vitro* hepatotoxicity of 4-AAP and reduce (~20%) the permeability of 4-AAP across a semi-permeable membrane. Molecular dynamic simulations generated a stable binding conformation between the PARA-061 peptide sequence and 4-AAP. In conclusion, we show that a phage display library can be used to identify peptide sequence-specific clones able to modulate the functional binding of a low molecular weight organic molecule. Such peptides may be expected to find utility in the next generation of hybrid polymer-based biosensing devices.

Keywords: Phage display; Peptide; 4-Acetamidophenol; Paracetamol; Low molecular weight organic molecules; Insoluble complexes

Phage display is a selection tool where a protein displayed on the surface of a bacteriophage as a fusion with one of the viral coat proteins, can be isolated from a vast library of similarly displayed proteins on the basis of its binding affinity to a target of interest [1,2]. The genetic information encoding the displayed protein is housed within the viral particle, allowing sequence identification of the displayed protein. The filamentous M13 phage is commonly used as the viral vector with the binding protein expressed primarily as a fusion with either coat protein VIII (pVIII) or protein III (pIII). The pIII libraries possess the advantage of identifying binding sequences of higher affinity, as the number of copies of the binding protein per phage particle is limited to a maximum of five.

Phage display libraries have been used to identify binding sequences in a range of applications primarily directed towards identifying peptides against protein targets [3]. In contrast no attention has been given to using phage display to identify peptide binders against non-proteinaceous low molecular weight organic moieties although there have been some limited reports of the interaction of peptide-phage libraries with non-proteinaceous surfaces such as metals [4,5] and plastic [6].

Peptides that bind low molecular weight organic species would have application as part of peptide-polymer constructs for use in sensing and neutralisation applications

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in the medical, environmental, and chemical sciences. However, a significant problem in phage display panning approaches against low molecular weight organic moieties is the immobilisation of the species without chemically occupying one of the limited recognition groups within the target molecule. In this study, we have used a cyclic peptide-phage display library to identify peptides that bind the low molecular weight organic molecule 4-acetamidophenol (4-AAP) otherwise known as paracetamol. We avoided occupancy of one of the few recognition groups on the 4-AAP molecule by panning against insoluble complexes rather than adopting a strategy that involves covalent linkage of the 4-AAP molecule to a stationary support. To further enrich for high affinity 4-AAP binders we included a deletion step that removed those phage peptide sequences that also bind the 4-AAP isomers, 2-acetamidophenol (2-AAP) and 3-acetamidophenol (3-AAP). The resulting peptide-phage particles were functional with respect to altering the diffusion capacity of solution 4-AAP and in modifying the cellular toxicity of 4-AAP in an *in vitro* hepatocyte model. This panning strategy may be expected to be useful when seeking to identify high affinity peptide motifs against low molecular weight organic species and where attachment to a stationary support is impractical.

Materials and methods

Materials. Sodium chloride (NaCl); IPTG and Xgal were obtained from Fisher Scientific (Loughborough, UK). Tween 20 was obtained from Amersham (Little Chalfont, UK). Agarose was obtained from Bioline (London, UK). Big dye v3.1 was obtained from Applied Biosystems (Warrington, UK). Luria-Bertani (LB) broth; LB agar; bovine serum albumin (BSA); 4-acetamidophenol; 2-acetamidophenol; 3-acetamidophenol; Trizma (Tris–HCl); *N*-acetylcysteine (NAC); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT); glycine–HCl and PEG-8000 were obtained from Sigma–Aldrich (Dorset, UK). Cell culture plastics were obtained from Corning Costar (High Wycombe, UK).

Cell lines. The human hepatocyte carcinoma cell line Hep3B (passages 18–23) was seeded at 3×10^4 cells/cm^2 and maintained in culture media comprising DMEM supplemented with 10% heat inactivated FBS and the antibiotics penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml). Hep3B cultures were maintained at 37 °C in a humidified atmosphere (5% CO_2/95% air) and culture media replenished every 48 h.

Phage display. The Ph.D.-C7C™ cyclised 7-mer disulphide constrained peptide-phage library (New England Biolabs, Hitchin, UK) was used for panning experiments against 4-AAP and its isomers 2-AAP and 3-AAP. This library represents 1.2×10^9 unique genotypes encoding random cyclised peptides fused to the pIII coat protein of the filamentous phage M13. The general motif of the displayed peptide is $^{N'}$AC-XXXXXXX CGGGS$^{C'}$ where X_7 represents a unique 7-mer peptide, the two cysteine residues cyclise the peptide and GGGS is a short amino acid spacer that links the peptide to the N terminus of the phage pIII coat protein.

A schematic of the panning experiments can be seen in Fig. 1, with all panning conducted against suspensions of insoluble complexes of 4-AAP, 3-AAP, or 2-AAP. Our panning using the peptide-phage library was divided into: (i) "Isolation strategy 1" involving four rounds of panning against 4-AAP (rounds 1–4): undertaken with the objective of isolating a pool of peptide-phage library members that bind with high affinity to 4-AAP, (ii) "Isolation strategy 2" involving two rounds of panning (rounds 5–6): undertaken to delete from the 4-AAP binding pool those phage also binding to the structural isomers 2-AAP and 3-AAP. The initial input to round 5 comprised phage from the final glycine elution step of panning round 4 in "Isolation strategy 1".

In "Isolation strategy 1" 4-AAP was suspended 500 mg/ml in TBS (50 mM Tris–HCl, pH 7.5; 150 mM NaCl) and incubated for 1 h at 37 °C with 1×10^{11} plaque forming units (pfu) of the Ph.D.-C7C™ phage library. This equates to incubation with ~83 copies of each of the 1.2×10^9 different phage library members. After incubation the 4-AAP was pelleted by centrifugation (10,500*g*) and the non-binding phage discarded. The 4-AAP pellet was washed 6× with TBS-T (TBS; 0.1% v/v Tween 20) with the 4-AAP pelleted, and supernatant discarded, between each wash. The 4-AAP binding phage were eluted with 3× glycine buffer elutions (0.2 M glycine–HCl, pH 2.2; 1 mg/ml BSA) again with the 4-AAP pelleted between each elution. The third glycine elution, containing the highest affinity binders, was collected and amplified (4.5 h) in a culture of *Escherichia coli*, and then purified by PEG precipitation; this phage population then served as input for the next round of panning. After four such rounds of panning within "Isolation strategy 1" an aliquot of the 4th round binders were gene sequenced using standard BigDye v3.1 chemistry on an ABI Prism 3100 Genetic Analyser. The remainder of the phage pool was amplified and served as the input into "Isolation strategy 2". In "Isolation strategy 2" a total of 1×10^{11} pfu of phage were exposed to a suspension of 2-AAP (500 mg/ml) for 1 h, following this the isomer was pelleted and the supernatant, containing non-binding peptide-phage, was transferred to a suspension of 3-AAP (500 mg/ml) for 1 h. The 3-AAP was subsequently pelleted and the supernatant containing non-binding peptide-phage transferred to a suspension of 4-AAP for 1 h. After this the 4-AAP was pelleted and washed 6× with TBS-T and 3× low pH glycine elutions as described above. The respective third glycine elution was collected and amplified as described for "Isolation strategy 1". A second panning round against 2-AAP, 3-AAP, and 4-AAP was repeated before gene sequencing to identify 4-AAP binding peptide sequences.

4-AAP permeability studies. The effect of the phage display identified 4-AAP binding peptide upon the diffusion of 4-AAP across a semi-permeable membrane was examined as one of our functional endpoints. Permeability studies were conducted in Franz cell diffusion apparatus with the donor and receiver chambers separated by a semi-permeable membrane (Nucleopore membrane, 100 nm pore size, Whatman Cat No. 110405). Phage were found to be incapable of penetrating this membrane to any great extent with, after 4 h, $<1 \times 10^{-6}$% of the 1×10^{11} phage loaded in the donor chamber penetrating to the receiver chamber.

Briefly, 100 μM 4-AAP in TBS was incubated in the donor chamber alone or with binding peptide-phage PARA-061, i.e., the selected 4-AAP binding peptide-phage clone, or with a random non-binding peptide-phage clone, NB-062. The phage were incubated in a 1:1 stochiometric ratio with 4-AAP molecules which equates to a maximum of five peptide copies per 4-AAP molecule. At predetermined time points following the start of the permeability experiment samples of the receiver chamber were collected and 4-AAP quantified. Receiver chamber sample volume was replenished with an equal volume of TBS. Samples were analysed for 4-AAP using high-pressure liquid chromatography (HP1050 analytical system, Agilent Technologies UK Ltd., West Lothian, UK; C18 column, Grace Vydac, Camforth, UK) with a mobile phase of 50:50 acetonitrile: H_2O at a flow rate of 1 ml min^{-1}. Injection volumes were 50μl with detection by UV/Vis at 550 nm.

The cumulative membrane transport of 4-AAP as a function of time was determined and the apparent permeability coefficients (ρ) ($\times 10^{-6}$ cms^{-1}) calculated using this equation:

$$\frac{dM}{dt} = p \times A \times \mathrm{Co} \quad (1)$$

where dM/dt represents the rate of change in cumulative mass of 4-AAP transferred from donor–receiver, A represents the area of the nucleopore membrane (0.95 cm^2) and Co is the original concentration of 4-AAP in the donor chamber (100 μM).

In vitro hepatotoxicity assay. As a further assessment of the functionality of the selected 4-AAP binding peptide-phage clone (PARA-061) we examined the *in vitro* modulation of 4-AAP induced hepatotoxicty. To assess 4-AAP *in vitro* hepatotoxicity we measured mitochondrial dehy-

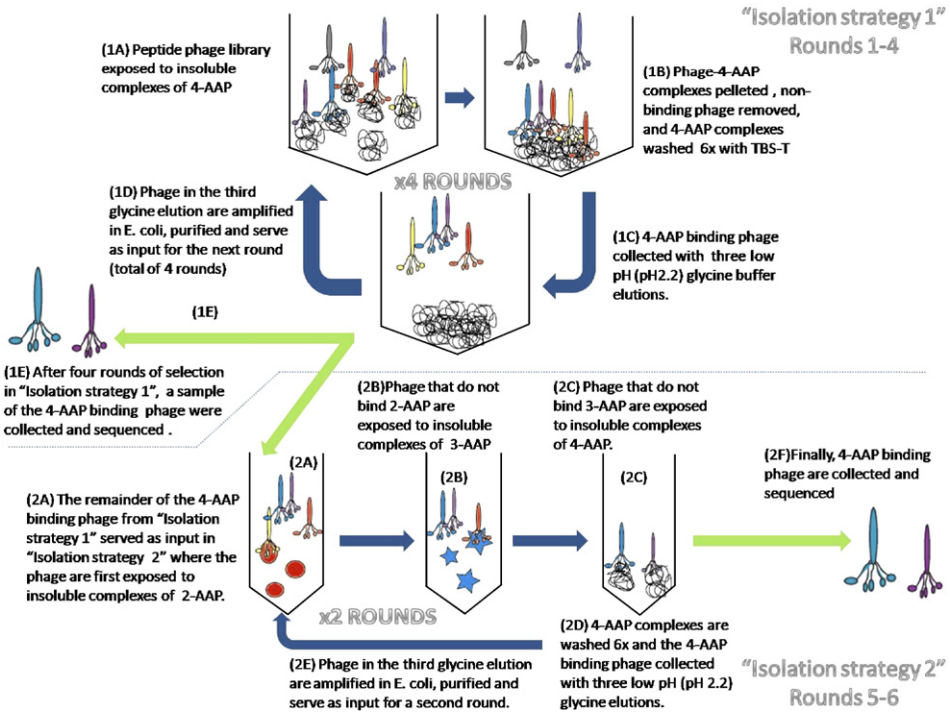


Fig. 1. Schematic of phage panning experiments. In "Isolation strategy 1" we sought to isolate a pool of clones enriched with peptide-phage that bind 4-AAP. The Ph.D.-C7C™ library was exposed to insoluble complexes of 4-AAP. The third glycine elution was collected and the phage amplified in *E. coli* and a further three rounds of panning completed (four rounds in total). Some peptide-phage collected in round 4 were sequenced. The remainder were used as an input for "Isolation strategy 2". In "Isolation strategy 2" peptide-phage that bound the isomers 2-AAP and 3-AAP were deleted from the 4-AAP binding pool by two rounds of exposure to insoluble complexes of 2-AAP and 3-AAP. Peptide-phage that did not bind 2-AAP or 3-AAP were collected and then exposed to 4-AAP to augment the 4-AAP binding population (rounds 5–6).

drogenase activity via the standard MTT assay as previously described [7]; MTT assays have commonly been used to evaluate the *in vitro* hepatotoxicty of 4-AAP [8]. Briefly, Hep3B cells were grown to confluence in 96 well plate format (5–7 days) and subsequently incubated with 4-AAP in the presence or absence of the 4-AAP binding peptide-phage clone PARA-061 or the non-binding peptide-phage clone NB-062. The phage virions were incubated in a 1:100 stochiometric ratio with 4-AAP molecules; this phage concentration reflecting the maximum phage pfu obtained by amplification in *E. coli*. Further incubations were conducted in the presence or absence of the reducing agent *N*-acetylcysteine (NAC; 10 mM). The therapeutic 'rescue' agent NAC has previously been shown to protect *in vitro* hepatocyte models from irreversible 4-AAP induced toxicity [9].

At predetermined time points of the incubation- treatments were removed, the cells washed and the media replaced with 250 μl of fresh media containing 100 μg of the substrate MTT. The plate was incubated for 4 h at 37 °C after which the media was removed and 200 μl of DMSO added to each well followed by the addition of 25 μl of Sorensen's glycine buffer (0.1 M glycine; 0.1 M NaCl equilibrated to pH 10.5) to dissolve the formazan product. The absorbance at 570 nm was measured using an Anthos htll microtiter reader (Labtech International, East Sussex, UK). Reduction in absorbance was indicative of reduced cell viability.

Molecular modelling and dynamic simulations. To explore the interaction of 4-AAP with our selected peptide PARA-061, we undertook molecular modelling studies. Molecular Dynamic simulations were performed with GROMACS 3.2 [10] and the GROMOS96 force field in a neutral pressure–temperature environment. The 4-AAP topology was built using the PRODRG server [11] and placed 10 Å away from the disulphide bridge of the peptide. The system was soaked in a triclinic water box and minimised using a steepest descent algorithm to remove unfavorable van der Waals contacts. The resulting system was then equilibrated via a 20 ps molecular dynamics simulation at 300K with restrained atoms. Finally, a 25 ns simulation was performed at 300K with a time step of 2 fs and hydrogen atoms constrained with a LINCS algorithm. Visualisation of the simulation trajectories was performed with the VMD software package, version 1.8.3 [12]. The MD simulation on peptide PARA-061 alone was performed for 10 ns using the procedure described above.

Results and discussion

Analysis of phage recovery and peptide sequences

The details of our panning scheme outlined in Fig. 1 enabled the selection of peptides that specifically bound 4-AAP. At the end of "Isolation strategy 1" round 4 with an input pfu of 1×10^{11} approximately 5000 phage were recovered. To augment our selection of 4-AAP binding phage we adopted a sequential isolation strategy ("Isolation strategy 2") and deleted those clones that also bound the isomers 2-AAP and 3-AAP. In the initial round of panning in "Isolation strategy 2", (round 5 of our overall scheme again with an input pfu of 1×10^{11}) we recovered only 100 phage clones. With again an input into round 6 of 1×10^{11} pfu an enrichment of the population of 4-AAP specific binders was evident by a 10-fold increase in recovered peptide-phage clones. After sequencing samples of the clones from "Isolation strategy 1" round 4 and "Isolation strategy 2" round 6, the sequence $^{N'}$ACNPN NLSHCGGGS$^{C'}$, assigned PARA-061, was identified in both isolation strategies suggestive of a presence in higher copy number (Table 1). This sequence was taken forward to analyse its functional binding affinity to 4-AAP. The

Table 1
Amino acid sequences of 4-AAP binding peptide-phage clones analysed on completion of Isolation strategies 1 and 2

Peptide sequences of phage clones binding to 4-AAP with unknown isomer binding status	Peptide sequences of phage clones binding to 4-AAP with 2-AAP and 3-AAP isomer binding peptide-phage clones deleted
Isolation strategy 1, round 4 (sample of five peptide-phage clones randomly selected from phage plaques)	Isolation strategy 2, round 6 (sample of 13 peptide-phage clones randomly selected from phage plaques)
AC-**NPNNLSH**-CGGGS (***PARA-061***)	AC-**NPNNLSH**-CGGGS (***PARA-061***)
AC-**KNFTHTD**-CGGGS	AC-**RAPSQTV**-CGGGS
AC-**TTASGAR**-CGGGS	AC-**DGNSRTQ**-CGGGS
AC-**TDLLPRH**-CGGGS	AC-**TTLTKTF**-CGGGS
AC-**PTAPLHM**-CGGGS	AC-**TLRSATA**-CGGGS
	AC-**SHLHSPL**-CGGGS
	AC-**ENTQKNS**-CGGGS
	AC-**SQGRLGQ**-CGGGS
	AC-**DRNGSNA**-CGGGS
	AC-**SQHSSRS**-CGGGS
	AC-**LNSHLQT**-CGGGS
	AC-**RTTSDAL**-CGGGS
	AC-**TSDWRLH**-CGGGS

PARA-061 is consistently present in Isolation strategies 1 and 2 and was taken forward to assess its 4-AAP binding neutralisation capacity. The sequence of the unique seven amino acid peptide displayed on the phage as a fusion with the pIII coat protein is indicated in bold letters.

non-binding phage NB-062, with sequence $^{N'}$ACHKSSHPQCGGGS$^{C'}$, was randomly selected from the first TBS-T wash in ''Isolation strategy 1'' round 1 as a negative control.

4-AAP permeability studies

To assess the capacity of the phage-peptide clone PARA-061 to bind 4-AAP, the permeability of 4-AAP across an inert semi-permeable membrane was determined following exposure of 4-AAP to the PARA-061 clone. The 4-AAP in solution was pre-exposed to PARA-061 for 30 min after which both the 4-AAP and the PARA-061 clone were loaded into the donor chambers of Franz cell diffusion apparatus. The permeability of 4-AAP in the presence of PARA-061 clone was compared to the permeability of 4-AAP alone, and importantly the permeability of 4-AAP pre-exposed to, and co-loaded with the non-binding peptide-phage clone NB-062. Non-binding peptide-phage clone had no effect ($p > 0.05$) upon 4-AAP permeability compared to the permeability of 4-AAP alone (Fig. 2). However, when 4-AAP was exposed to peptide-phage clone PARA-061, the permeability of 4-AAP was significantly reduced ~20% ($p < 0.05$) compared to both 4-AAP alone and to 4-AAP exposed to the non-binding clone NB-062.

In vitro hepatotoxicity assay

The functionality of the 4-AAP binding clone PARA-061 was further assessed by examining its effectiveness in

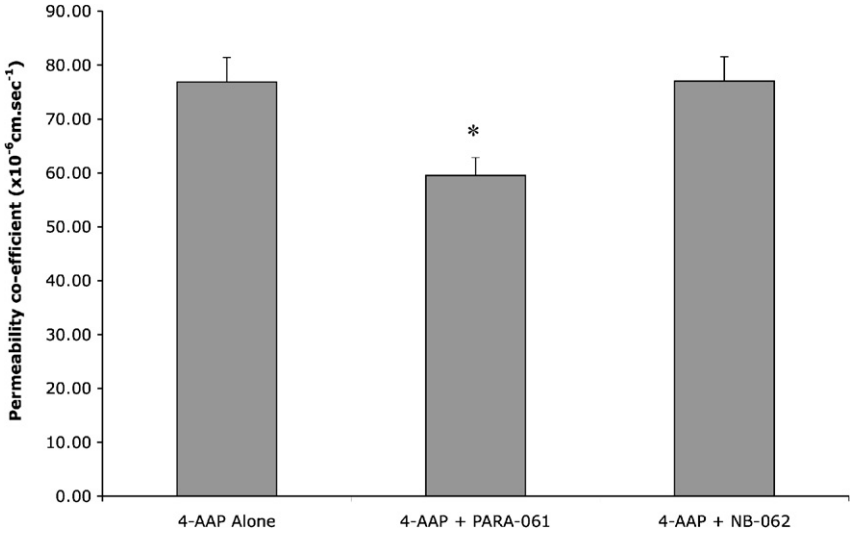


Fig. 2. Permeability of 4-AAP through a nucleopore membrane (100 nm pore size) placed between the donor and receiver chambers of a Franz cell. Permeability experiments showed that PARA-061, but not a non-binding peptide-phage clone NB-062, significantly ($p < 0.05$) reduced 4-AAP permeability compared to 4-AAP alone. *Signifies statistically significant difference ($p < 0.05$) compared to any of the other treatments. Data are mean ± SD ($n = 4$).

preventing hepatocyte toxicity. The first sign of 4-AAP toxicity in our *in vitro* hepatocyte model occurred after 5 h incubation with 4-AAP at a concentration of 40 mM (Fig. 3A), which could be prevented by co-incubation with the thiol containing reducing agent *N*-acetylcysteine (10 mM). Longer 4-AAP exposure times resulted in a progressively greater loss in cell viability that could not be prevented by co-treatment with NAC. Therefore, we used an exposure of 40 mM 4-AAP for 5 h as an optimised experimental condition to test the effectiveness of the PARA-061 clone in minimising loss of 4-AAP induced hepatocyte viability. Co-incubation of the phage clone PARA-061 and 4-AAP, in a 1:100 ratio prevented loss in hepatocyte viability, with cell survival recorded to be similar ($p > 0.05$) to cells treated with combined 4-AAP and NAC or to respective control cells not exposed to any treatment (Fig. 3B). This contrasts with the significant ($p < 0.05$) loss in cell viability seen when non-binding phage clone NB-062 was co-incubated with 4-AAP (40 mM) for 5 h (Fig. 3B).

Molecular modelling and dynamic simulations

To probe the potential binding interactions of 4-AAP to the PARA-061 peptide, a series of molecular dynamic simulations were performed. As the simulation time progressed, periods of low energy ligand interaction became

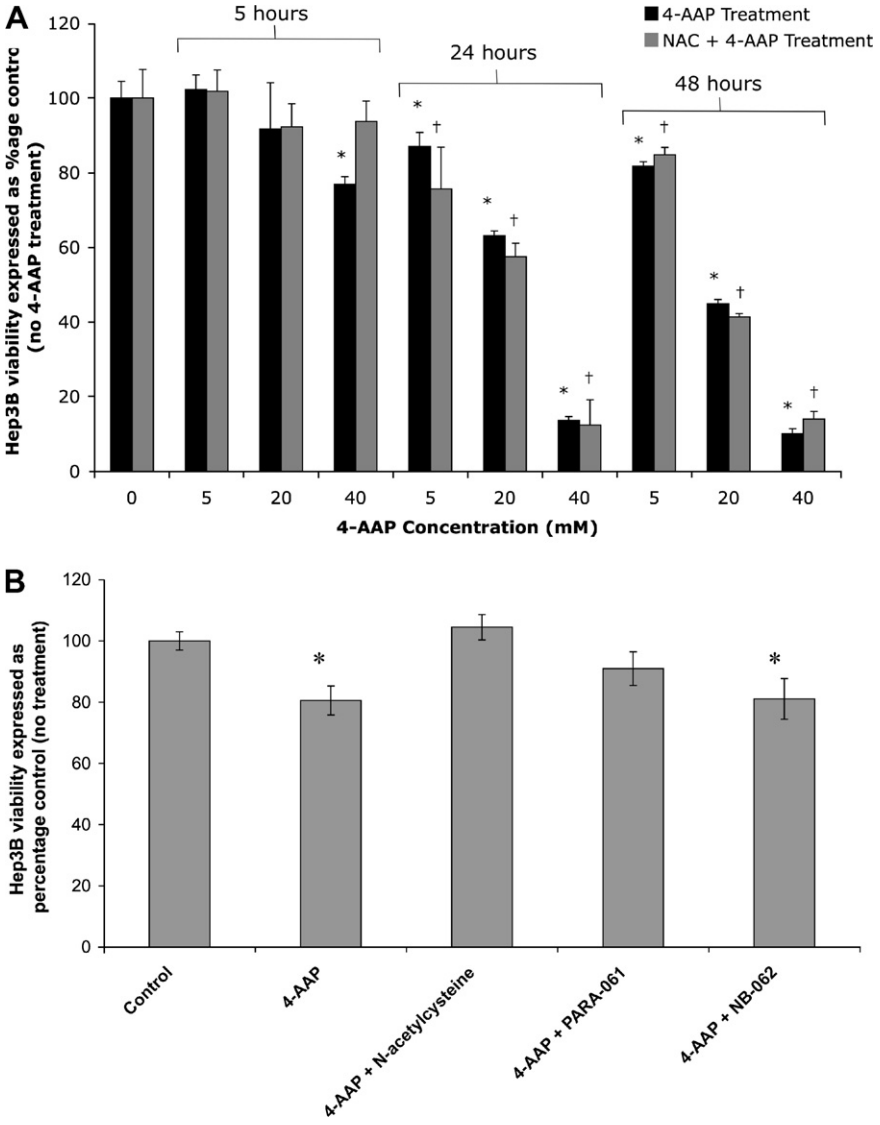


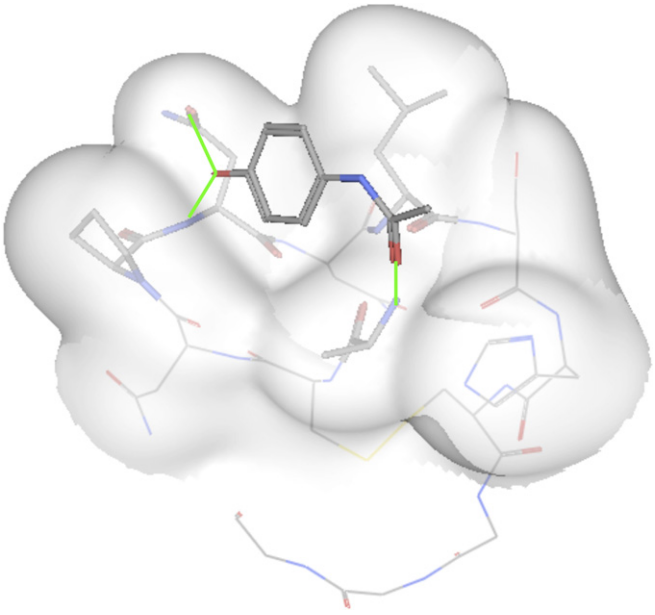
Fig. 3. Mitochondrial dehydrogenase activity of Hep3B cells exposed to 4-AAP with results expressed as mean absorbance at 570 nm as percentage control where control is either no treatment or exposure to *N*-acetylcysteine (NAC) alone. (A) 4-AAP is toxic to Hep3B cells both in a time and concentration dependent manner. Exposure of Hep3B cells for 5 h to 40 mM of 4-AAP led to a significant ($p < 0.05$) 20% reduction in cell viability that was prevented by co-incubation with NAC. Data are means ± SD ($n = 4$). *Signifies statistically significant difference ($p < 0.05$) compared to no treatment cells. †Signifies statistically significant difference ($p < 0.05$) compared to cells exposed to NAC alone. (B) In Hep3B cells exposed to 4-AAP (40 mM for 5 h) and co-incubated with peptide-phage PARA-061, 4-AAP induced hepatotoxicity was prevented. In contrast, co-incubation with a non-binding peptide-phage clone NB-062 did not prevent 4-AAP induced hepatotoxicity. Data are means ± SEM for three separate experiments where $n = 6$–8 for each experiment. *Signifies statistical difference ($p < 0.05$) compared to no treatment control and binding phage clone PARA-061.

evident. Two intervals were of particular interest: from 12.5 to 17.5 ns and from 17.5 to 22.5 ns. The latter reflected the 4-AAP molecule interacting mainly with the GGGS spacer, a part of the peptide that would be less accessible when attached to the pIII protein on the phage virion and a part of the peptide common to all members of the peptide-phage library. Between 12.5 and 17.5 ns the 4-AAP bound at regions of the sequence unique in the library. This binding was stable, forming up to three hydrogen bonds at any one time: two between the asparagine of the peptide and the hydroxyl group of 4-AAP, and one between the alanine of the peptide and the carbonyl of 4-AAP. Further, the aromatic ring of 4-AAP was positioned between the hydrophobic residues proline and leucine within the peptide (Fig. 4A). In support of simulation binding trajectories observed between 12.5 and 17.5 ns, the average number of hydrogen bonds between the peptide and 4-AAP was also at its greatest (Fig. 4B). This specific binding conformation is stable for several ns during the simulation in accordance with the energy results obtained.

In conclusion, identifying peptide motifs against low molecular weight organic molecules can be expected to have numerous downstream applications in biomedical and environmental sensing and monitoring. However, a significant limitation in exploiting low molecular weight organic molecules as target for phage display is how such molecules can be immobilised or captured for the phage panning process without chemically occupying one of the limited functional groups within the molecule or introducing a significant chemical functionality such as linker groups or tags.

Insoluble complexes of low molecular weight molecules whilst having a heterogeneous and non-defined surface should nevertheless display the various functional groups

A



B

Docking simulation time steps (nanoseconds)	Average ligand interaction energy (kjoules.mol^{-1})	Average number of hydrogen bonds between ligand (4-AAP) and peptide (PARA-061)
0 – 2.5	-1.3888648	0.824
2.5 – 5.0	1.72451634	0.396
5.0 – 7.5	3.92685093	0.372
7.5 – 10.0	-1.9904484	0.708
10.0 – 12.5	-2.103934	0.454
12.5 – 15.0	-3.8409059	0.834
15.0 – 17.5	-4.0036025	0.586
17.5 – 20.0	-6.2764456	0.126
20.0 – 22.5	-6.0959617	0.406
22.5 – 25.0	-3.2946138	0.050

Fig. 4. *In silico* molecular dynamic simulations of the interaction of 4-AAP with peptide $^{N'}$ACNPNNLSHCGGGS$^{C'}$ (sequence in PARA-061 clone). (A) Examination of the simulation trajectory between 12.5 and 17.5 ns showed that PARA-061 stably binds 4-AAP forming up to three hydrogen bonds, two between the asparagine and the hydroxyl group of 4-AAP, and one between alanine and the carbonyl of 4-AAP. The aromatic ring of the 4-AAP molecule is suitably positioned between the hydrophobic residues proline and leucine. (B) Binding energy and average number of hydrogen bonds between 4-AAP and $^{N'}$ACNPNNLSHCGGGS$^{C'}$ peptide sequence. Between 12.5 and 17.5 ns the average ligand interaction energy is low and the average number of hydrogen bonds is high indicating stable binding.

of the parent molecule and therefore represent a target against which phage display selection can be undertaken. In this work, we undertook peptide-phage panning against insoluble complexes of 4-AAP. The 4-AAP binding peptide-phage clone selected from our Isolation strategies retained binding functionality with respect to free 4-AAP molecules in solution as assessed by both functional modulation of 4-AAP diffusion across a semi-permeable membrane and by modulation of 4-AAP hepatotoxicity in an established *in vitro* model. The selected peptide also displayed low interaction energy with the 4-AAP molecule in molecular dynamic simulations.

We have shown how it is possible to generate functional binding peptides from a phage display library against low molecular weight organic species. Whilst the strategy of using insoluble complexes as the panning target may or may not prove appropriate for a wide range of similar molecules, peptides that are functional with respect to such molecules and that can be identified by display approaches is an original finding. Such peptides will serve as recognition motifs within the next generation of hybrid polymeric molecular sensors or therapeutics.

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