BIOCHEMICAL PHARMACOLOGY 75 (2008) 1743–1750

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A facile method to screen inhibitors of protein–protein interactions including MDM2–p53 displayed on T7 phage

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ARTICLE INFO

Article history:
Received 10 October 2007
Accepted 28 January 2008

Keywords:
Phage display
Nutlin 3
Dehydroaltenusin
Protein–protein interaction
p53
MDM2

ABSTRACT

Protein–protein interactions are essential in many biological processes including cell cycle and apoptosis. It is currently of great medical interest to inhibit specific protein–protein interactions in order to treat a variety of disease states. Here, we describe a facile multiwell plate assay method using T7 phage display to screen for candidate inhibitors of protein–protein interactions. Because T7 phage display is an effective method for detecting protein–protein interactions, we aimed to utilize this technique to screen for small-molecule inhibitors that disrupt these types of interaction. We used the well-characterized interaction between p53 and MDM2 and an inhibitor of this interaction, nutlin 3, as a model system to establish a new screening method. Phage particles displaying p53 interacted with GST–MDM2 immobilized on 96-well plates, and the interaction was inhibited by nutlin 3. Multiwell plate assay was then performed using a natural product library, which identified dehydroaltenusin as a candidate inhibitor of the p53–MDM2 interaction. We discuss the potential applications of this novel T7 phage display methodology, which we propose to call 'reverse phage display'.

1. Introduction

Protein–protein interactions have been recognized to be essential in many biological events including cell cycle and apoptosis [1–4]. The tumor suppressor, p53, is known to interact with the E3 ubiquitin ligase, MDM2. The cellular level of p53 is tightly controlled by degradation *via* the ubiquitin–proteasome pathway [5]. However, the mechanism that controls the level of p53 can be disrupted in cells over-expressing MDM2, which includes some cancer cells [5]. Eventually, such cancer cells may escape from p53-dependent cell cycle arrest and apoptosis, resulting in cell survival and proliferation. Because such protein–protein interactions have an impact on a number of different biological processes, disruption of these interactions can be a useful strategy towards developing pharmaceutical agents that possess novel modes of action [6–10]. Vassilev et al. reported that nutlin 3 is a small-molecule inhibitor of the p53 and MDM2 interaction, which activates the p53 pathway in cancer cells leading to cell arrest, apoptosis and growth inhibition [9]. Furthermore, the small-molecule 4EGI-1 inhibits protein–protein interaction between elF4E and elF4G, which interferes with cap-dependent translation [10]. These two examples demonstrate the interest within the pharmaceutical sector in identifying small-molecule inhibitors of protein–protein interactions.

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Abbreviations: PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; GST, glutathione S-transferase; *p*NPP, *p*-nitrophenyl phosphate; CBB, Coomassie brilliant blue R250; IPTG, isopropyl-beta-D-galactoside; PFU, plaque forming unit.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.01.020

Phage display cloning is a useful method for analyzing protein–protein interactions [11]. Heterogeneous and diverse proteins or peptides, which are fused to T7 coat protein 10B [12,13] can be displayed on T7 phage particles. Upon infection and plating of *Escherichia coli*, T7 phage particles form plaques after only 3 h. Furthermore, analysis of protein–protein interactions using phage requires neither specialist equipment, such as a surface plasmon resonance (SPR), nor time consuming analyses, as is the case with the yeast 2 hybrid system [4,14]. These characteristic features of T7 phage display cloning make it suitable as a multiwell plate platform for identifying novel protein–protein interactions. Morohashi et al. recently developed high-throughput screening system based on the T7 phage display method [15]. However, this procedure depended on peptide-small-molecule interactions rather than protein–protein interactions.

Here we aimed to utilize T7 phage display for the screening of candidate inhibitors for protein–protein interactions. The p53–MDM2 interaction and the inhibitor nutlin 3 [9] were chosen to act as a model system to test the reliability of this novel screening procedure. We engineered T7 phage particles to display p53 and constructed the p53–MDM2 interaction in a 96-well plate format. We then verified that nutlin 3 inhibited the interaction. Based on these results, the multiwell plate assay system was used to identify inhibitors from a natural product library. Our screening identified dehydroaltenusin as a candidate inhibitor of the p53–MDM2 interaction. In addition, we discuss the potential applications of this T7 phage display method for multiwell plate assay of small-molecule libraries to identify candidate inhibitors of protein–protein interactions.

2. Materials and methods

2.1. Construction of T7 phage displaying p53

A fragment of p53 cDNA, encoding residues 12–36, was amplified by PCR using p53 forward (GGGAATTCGGATCCTAGCGTCGAGCCCCCTCTGAGTCAGGAAACAT) and a p53 reverse (CCAAGCTTTTACGGCAAGGGGGACAGAACGTTGTTTTCAGGAATAGTTTCCATAGGTCTGAAAATGTTTCCTGAC) primer. The p53 forward and p53 reverse primers include an *Eco*RI and a *Hind*III endonuclease recognition site, respectively (highlighted by underline). The PCR product was digested with *Eco*RI/*Hind*III and cloned into the corresponding sites on the T7 Select10-3b vector (Novagen, Wisconsin, WI). The DNA was then packaged into T7 phage particles using an *in vitro* T7 packaging extract (Novagen). The packaging solution was amplified once in isopropyl-beta-D-galactoside (IPTG) induced *E. coli* BLT5615 strain (Novagen). DNA sequencing confirmed that the fragment of p53 gene was fused in-frame to the C-terminus of the T7 coat protein 10B. Manipulations of T7 phage and the growth media are detailed in the T7 Select System manual (Novagen) [12] and the work of Krumpe et al. [13].

2.2. Construction of the GST–MDM2

The fragment of MDM2 cDNA, containing residues 25–117, was amplified from a human breast cancer cDNA library using an MDM2-forward (GGGGATCCGAGACCCTGGTTAGACCAAAGCCAT) and an MDM2-reverse (CCGAATTCTTATGAATTTTCTTCCTGCTGATTGACT) primer, containing a *Bam*HI and an *Eco*RI recognition site, respectively (highlighted by underline). The PCR product was digested with *Bam*HI/*Eco*RI and cloned into the corresponding sites of pGEX6p-1 (GE Healthcare, Piscataway, NJ). DNA sequencing confirmed that the fragment of p53 gene was fused in-frame to the C-terminus of the GST encoding gene of pGEX6p-1. GST–MDM2 was expressed in *E. coli* BL21(DE3) using the constructed plasmid, pGMDM2. A single colony of transformed *E. coli* was inoculated to prepare a seed culture of LB medium (peptone 10 g/l, yeast extract 5 g/l and NaCl 5 g/l with 50 mg/l ampicillin) and incubated at 37 °C overnight with vigorous shaking. A 20 ml aliquot of seed culture was used to inoculate 500 ml LB medium. The culture was incubated at 37 °C with vigorous shaking until the OD_{600} reached 0.5–1.0. IPTG (final concentration 1 mM) was then added to the culture to induce heterologous expression of GST–MDM2. The culture was incubated for a further 3 h before harvesting the cells by centrifugation (5000 × *g* for 10 min at 4 °C). Harvested cells were resuspended in PBS and disrupted by ultrasonic treatment using a Branson sonifier (Branson Ultrasonics, Danbury, CT). The cell-free extract was centrifuged at 14,000 × *g* for 20 min at 4 °C, and the supernatant was then filtered through a Millex HV 0.45 μm filter unit (Millipore, Danvers, MA). The clarified solution was then loaded onto a 1 ml GSTrap FF (GE Healthcare). After washing the column with buffer, bound GST–MDM2 was eluted using glutathione. All chromatography was carried out using an AKTA FPLC system (GE Healthcare). The purity of GST–MDM2 was assessed by SDS–polyacrylamide gel electrophoresis using a 12.5% gel [16]. A single band corresponding to the anticipated molecular weight of GST–MDM2 was visible after staining the gel with Coomassie brilliant blue R250 (CBB). Glutathione in the purified GST–MDM2 was removed by gel filtration chromatography using a PD10 column (GE Healthcare) according to the manufacture's instructions.

2.3. Binding assay between GST–MDM2 and p53 displayed on T7 phage particles

A 200 μl aliquot of protein solution (~0.2 mg/ml in PBS) was added to each well of a test plate (128 mm × 85 mm × 17 mm, Techno Plastic Products AG, Trasadingen, Switzerland) to immobilize GST–MDM2. The solution was incubated for ~30 min at room temperature with gentle shaking [17]. After washing each well several times with 200 μl of PBS, a 200 μl aliquot of either wild-type or p53 displaying phage particles (~10^{10}) was added. The mixture was then incubated for 30–60 min at room temperature in an orbital shaker. Each well was washed five times with 200 μl PBS to remove non-specifically bound phage. The bound phage particles were then eluted with 200 μl of 1% SDS in PBS for ~30 min at room temperature with gentle shaking. Each sample was used to inoculate a lawn of *E. coli* BLT5615 strain (Novagen) on LB agar medium in a dish (100 mm × 15 mm, BD, NJ) or a square plate (140 mm × 100 mm × 15 mm, Nissui, Tokyo, Japan). The number of phage particles in each well was determined by counting the plaques formed on the dishes or plates. The plaque assay method was followed by reference to the T7

Select System manual (Novagen) [12] and Morohashi et al. [18].

2.4. Detection of p53 displayed on T7 phage particles

Wild-type (WT) phage particles and phage particles displaying p53 ($\sim 10^8$ particles, each) were boiled in Laemmli sample buffer for 5 min and then analyzed on duplicate 12.5% SDS–polyacrylamide gels [16]. One gel was stained with CBB and the other gel was blotted onto a PVDF membrane using a semidry blotting apparatus (Trans-blot SD semi-Dry electrophoretic transfer cell; Bio-Rad laboratories, Hercules, CA). An anti-p53 antibody sc263 (Santa Cruz Biotechnology, Santa Cruz, CA) and an alkaline phosphatase conjugated anti-mouse antibody (Sigma–Aldrich, St. Louis, MO) was used to identify the recombinant fused protein. Bands were visualized by chemi-fluorescence on a CDP star (GE Healthcare).

2.5. Inhibition assay by anti-p53 antibody

A plaque assay method was performed for the binding between immobilized GST–MDM2 and p53 displayed on T7 phage particles. A 200 μl aliquot of protein solution (~0.2 mg/ml in PBS) was added to each well of a test plate (128 mm × 85 mm × 17 mm, Techno Plastic Products AG). Immobilization of GST–MDM2 was achieved by simply incubating the plate for ~30 min at room temperature with gentle shaking [16]. Each well was washed several times with 200 μl of PBS before adding 180 μl of WT or p53 displaying phage particles ($\sim 10^{10}$). A solution (20 μl) of anti p53 antibody (4 μg in 20 ml stock buffer; Santa Cruz) was then immediately added to the MDM2-immobilized wells. The mixture was incubated for 30–60 min at room temperature on an orbital shaker. Each well was washed with buffer and bound phage particles were eluted with 200 μl of 1% SDS. The number of phage particles in each well was then determined.

2.6. Inhibition assay by small molecules

Inhibition of the binding between immobilized GST–MDM2 and p53 displayed on T7 phage particles was assessed using a plaque assay. After immobilizing GST–MDM2 on a 96 well plate, 200 μl of T7 phage particles ($\sim 10^{10}$) was added to each well. A solution (2 μl) of nutlin 3 (0.0016, 0.016, 0.16 and 1.6 mM) in DMSO was immediately added to each well to give a final concentration of 0.016, 0.16, 1.6 and 16 μM. The plate was then incubated for 30–60 min at room temperature with gentle shaking. After incubation, each well was washed and bound phage eluted with 200 μl of 1% SDS. The number of phage particles in each well was then determined.

Each natural product in our library including both known and unknown compounds (about 70) was isolated from the cultured liquid media of fungi and Mosses, which were collected by Sugawara and collaborators in Tokyo University of Science. For one primary screening, a 2 μl aliquot of natural product in chloroform (10 mM) was added to give a final concentration of 100 μM, after addition of 200 μl of T7 phage ($\sim 10^{10}$) to each well containing immobilized GST–MDM2. Each natural product in chloroform and each 2 μl triplicate solutions of chloroform as a negative control and of nutlin 3 in DMSO as a positive control were used for one primary screening (triplicate blank wells without any natural products were used as negative control). The plate was incubated for 30–60 min at room temperature with gentle shaking before determining the number of bound phage particles per well as described earlier. After washing and eluting with 1% SDS solution, the number of plaques in each elution was adequately diluted to 10, 100 and 1000 times by using multi-channel pippeter to count precisely. Primary screening identified dehydroaltenusin and the other compounds as candidate inhibitors. Candidate compounds were dissolved in DMSO for precisely comparing with nutlin 3 in DMSO as a positive control and eliminating false positive compounds and both 2 μl triplicate solutions of the compounds and nutlin 3 were tested. Negative control wells contained 2 μl DMSO only. Tests were three times repeated.

2.7. Detection of immobilized GST–MDM2$_{25–117}$

The stability of the immobilized GST–MDM2$_{25–117}$ on a 96-well plate was tested as follows. A solution (200 μl) of GST–MDM2$_{25–117}$ (about 0.2 mg/ml) in PBS was added to each well of a test plate (Techno Plastic Products AG) to immobilize the protein. The mixture was allowed to incubate for about 30 min at room temperature with gentle shaking. Each well was then washed several times with a solution of PBS (200 μl) before addition of nutlin 3 (final concentration 1 nM to 10 μM), dehydroaltenusin (final concentration 10 nM to 100 μM) or SDS (final concentration 1 μM to 10 mM). After addition of each solution (200 μl) of the compounds in PBS, the plate was incubated for about 60 min with gentle shaking at room temperature. After incubation, each well was washed five times with 200 μl of PBS. A solution of mouse anti-GST antibody in PBS (200 μl used at 1:1500 dilution, Sigma–Aldrich) was added to each well and the mixture was incubated for 30 min with gentle shaking at room temperature. The solution in each well was then discarded and a solution (200 μl) of rabbit anti-mouse IgG alkaline phosphatase conjugated IgG antibody (used at 1:1500 dilution; Sigma–Aldrich) was added. The plate was incubated for 30 min with gentle shaking at room temperature. After incubation, each well was washed three times with a solution of PBS (200 μl). A solution of 14.2 mM *p*NPP (100 μl, Nacalai Tesque, Kyoto, Japan) in PBS was added to each well and incubated for about 30 min at room temperature. The activity of alkaline phosphatase was then terminated by addition of an aqueous 1 M NaOH (15 μl) to each well. The absorbance of each well was determined at 405 nm using a spectrophotometer (Wallac arvo sx1420 multilabel counter; PerkinElmer, Chicago, IL). Non-immobilized GST–MDM2$_{25–117}$ wells were used as negative control.

3. Results

3.1. Construction of MDM2$_{25–117}$–p53$_{12–36}$ interaction on T7 phage particles

The binding of MDM2$_{25–117}$ and p53$_{12–36}$ has been reported to have a dissociation constant of $\sim 10^{-7}$ (Fig. 1A) [8,19]. Here, the binding of MDM2$_{25–117}$ and p53$_{12–36}$ has been employed to develop a phage display screening system for protein–protein

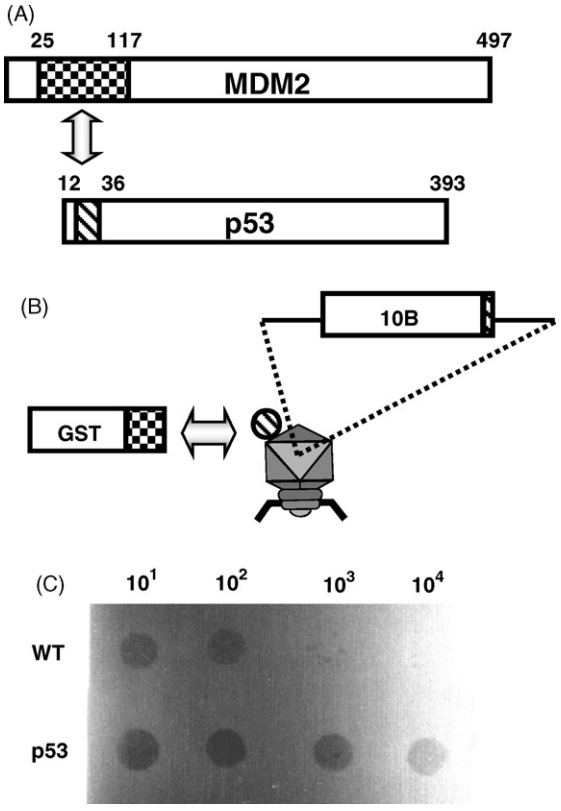

Fig. 1 – Schematic diagram for the construction of $MDM2_{25–117}$–$p53_{12–36}$ interaction on T7 phage particles and single clone check analysis. (A) The binding regions of p53 (12–36) and MDM2 (25–117) are represented in the slash box and checker box, respectively. The binding region of MDM2 (25–117) was expressed as a GST fusion protein and p53 (12–36) was displayed on a T7 phage particle; (B) model of the interaction between GST–MDM2 (open and checker box) immobilized on 96 wells and p53 displayed on T7 phage particles (slash open); (C) serial dilutions (10^{-1} to 10^{-4}) of recovered phage particles of WT phage (10^6 pfu/ml) and p53 displaying phage (10^8 pfu/ml) were spotted onto a lawn of growing *Escherichia coli*. After infection, the number of plaques formed was determined. WT represents T7 phage of wild type phage; p53 represents T7 phage expressing $p53_{12–36}$.

interactions. The N-terminal peptide of $MDM2_{25–117}$ was prepared as a GST fusion protein and immobilized on a 96-well plate [16]. The N-terminal peptide of $p53_{12–36}$ was displayed on the T7 phage (Fig. 1B). First, we compared the number of the $p53_{12–36}$ displayed phage particles recovered from the $MDM2_{25–117}$ immobilized plates with those of the wild type phage particles to confirm specific binding of $p53_{12–36}$ displayed phages with the GST–$MDM2_{25–117}$. Our results show that the number of $p53_{12–36}$ displayed phage particles bound to $MDM2_{25–117}$ immobilized plates was one hundred times greater than that of T7 wild type (WT) phage particles (Fig. 1C). However, when the experiment was repeated using control plates, which were free of immobilized GST–$MDM2_{25–117}$ fused protein, the number of bound WT phage particles and $p53_{12–36}$ displayed phage particles was the same (data not shown). These results confirm the anticipated interaction between plate immobilized GST–$MDM2_{25–117}$ protein and $p53_{12–36}$ displayed on the T7 phage particles. Next, we examined the interaction of GST–$MDM2_{25–117}$ and $p53_{12–36}$ using an antibody to p53. Western blot analysis showed that an antibody raised against the N-terminus of p53 cross reacted with a protein extract of phage particles displaying $p53_{12–36}$ but not with a protein extract of WT phage particles (Fig. 2A). In this experiment, two cross-reacting species in the extract from $p53_{12–36}$ displayed phage particle were observed as shown in Fig. 2A (right). It was unclear whether either of these cross-reacting bands corresponded to $p53_{12–36}$ fused to the coat protein 10B of the T7 phage particle. Thus, we investigated whether the antibody against p53 inhibited the interaction between plate-immobilized GST–$MDM2_{25–117}$ and T7 phage particles displaying $p53_{12–36}$. The number of wild type phage particles recovered from plates was unchanged by the addition of the p53 antibody. The immobilized amounts of GST–MDM2 protein can be varied by each lot and/or each experiment and PFU can be also influenced. Thus the assays performed here were carried out in duplicate or triplicate samples and repeated two or three times experiments with the presence of both positive and negative controls. Since the comparative relationships could be same, therefore, the obtained results were conclusively same in each experiment. By contrast, the number of recovered phage particles displaying $p53_{12–36}$ decreased by 90% after addition of p53 antibody (Fig. 2B). Taken together, our results verify the expected interaction between plate-immobilized GST–$MDM2_{25–117}$ and $p53_{12–36}$ displayed on T7 phage particles.

3.2. *Inhibition of the interaction between GST–$MDM2_{25–117}$ and $p53_{12–36}$ on T7 phage particles by nutlin 3*

Nutlin 3 is known to inhibit the interaction of MDM2 and p53 (Fig. 3A) [9]. We examined the effect of nutlin 3 on the interaction of plate-immobilized GST–$MDM2_{25–117}$ and $p53_{12–36}$ displayed on T7 phage particles prior to screening for inhibitors from a natural product library. A solution of T7 phage particles displaying $p53_{12–36}$ was added to each well containing immobilized GST–$MDM2_{25–117}$ protein followed by a solution containing various concentrations of nutlin 3. The number of the recovered phage particles displaying $p53_{12–36}$ decreased as the concentration of nutlin 3 increased. These results indicate that the interaction of GST–$MDM2_{25–117}$ protein with $p53_{12–36}$ is inhibited by nutlin 3. The lack of $p53_{12–36}$ fused peptide in the coat protein of wild type T7 phage particles prevented the binding to plate-immobilized $MDM2_{25–117}$ protein. As anticipated, the numbers of recovered phage particles from $MDM2_{25–117}$ immobilized plates was unchanged by the addition of the inhibitor, nutlin 3 (Fig. 3B). These results indicated that our novel screening procedure was likely to detect small-molecule inhibitors of the interaction between $MDM2_{25–117}$ and $p53_{12–36}$.

3.3. *Screening inhibitors from a natural product library*

The T7 phage display method is well suited to a multiwell plate assay platform. Therefore, we developed a novel procedure

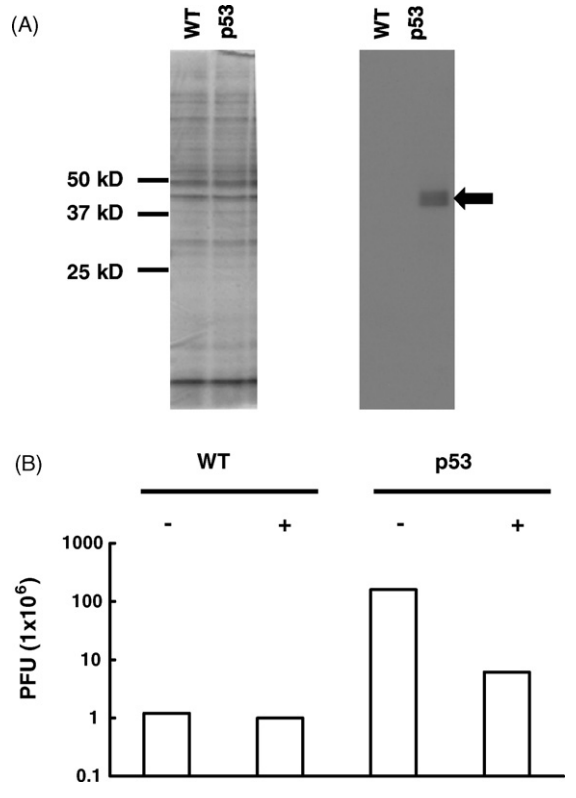

Fig. 2 – Detection of 10B-fused fragment of p53 on T7 phage particles and inhibition of the interaction of the GST–MDM2$_{25–117}$ and the p53$_{12–36}$ using an antibody against p53. (A) Crude extracts of proteins from about 10^8 of T7 phage particles were analyzed on 12% SDS-PAGE. WT lane, extract from wild type phage; p53 lane, extract of phage expressing p53$_{12–36}$. Left: the developed gel was stained using Coomassie brilliant blue R250, right: Western blot analysis to detect the 10B-fused fragment of p53$_{12–36}$ on T7 phage particles using an anti-p53 p53 antibody. The arrow highlights the band corresponding to 10B-fused p53$_{12–36}$. (B) Y-axis represents the number of PFU ($\times 10^6$). The number of phage particles recovered from GST–MDM2$_{25–117}$ immobilized plates was determined using the plaque assay method [18]. The + and − in the column represents the presence or absence of an anti-p53 antibody, respectively. All experiments were repeated twice to confirm the reproducibility of the inhibition assay.

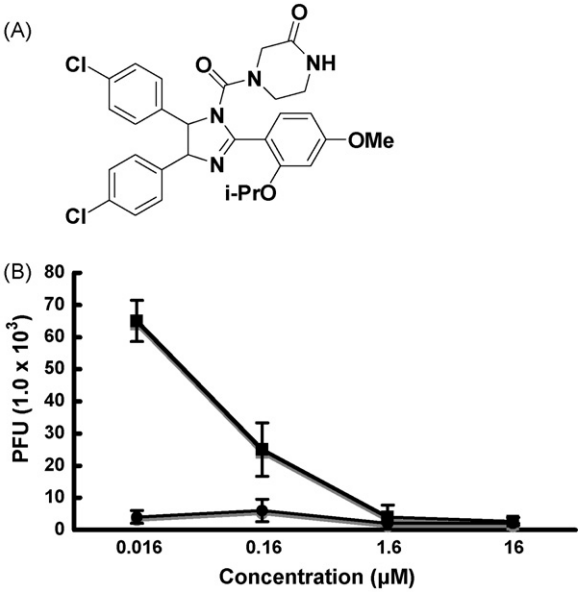

Fig. 3 – Structure of nutlin 3 and its inhibitory assay. (A) Structure of nutlin 3, (B) Y-axis shows the number of T7 phage particles displaying p53$_{12–36}$ that were recovered from plates containing immobilized GST–MDM2$_{25–117}$ in the presence (closed boxes) or absence (closed circles) of nutlin 3. The concentration of inhibitor is shown in the X-axis. The data are averages ± S.D. of triplicate determinations from a single experiment that has been repeated twice with identical results.

based on phage display to screen for small-molecule inhibitors of protein–protein interactions (Fig. 4A). The immobilized GST–MDM2$_{25–117}$ protein in a 96-well plate was treated with a chemical SDS or an inhibitor nutlin 3, and then it was washed to determine the amount of remaining protein with ELISA to make sure the stability against the treatments by inhibitors. After immobilization of GST–MDM2$_{25–117}$ protein on a 96-well plate, a solution (2 μl) of SDS (1 M, 100, 10, 1 mM or 100 μM) in PBS was added to each well to give a final concentration of 10, 1 mM, 100, 10 or 1 μM. The plate was incubated for 60 min before washing each well several times with PBS. The amount of remaining GST–MDM2$_{25–117}$ protein in each well was then determined by ELISA (Table 1). The amount of GST–MDM2$_{25–117}$ protein detected in the wells was reduced after addition of

Table 1 – The stability of the immobilized GST–MDM2$_{25–117}$ on a 96-well plate

Drug	10 nM	100 nM	1 μM	10 μM	100 μM	1 mM	10 mM
SDS	n.d.	n.d.	0.915	0.926	0.886	0.384	0.362
Nutlin 3	0.915	0.892	0.906	1.06	n.d	n.d	n.d
Dehydroaltenusin	0.942	0.862	0.921	0.897	1.039	n.d.	n.d.

A solution (200 μl) of GST–MDM2$_{25–117}$ (about 0.2 mg/ml) in PBS was added to each well of a test plate to immobilize the protein. After incubation, each well was washed several times with PBS (200 μl). After addition of each solution (200 μl) of nutlin 3 (final concentration 1 nM to 10 μM), dehydroaltenusin (final concentration 10 nM to 100 μM) or SDS (final concentration 1 μM to 10 mM) in PBS, each plate was incubated and then washed with PBS. A solution of mouse anti-GST antibody in PBS was added to each well and the mixture was incubated and then discarded. A solution (200 μl) of rabbit anti-mouse IgG alkaline phosphatase conjugated IgG antibody was added into each well, incubated and then washed with PBS. After a solution of 14.2 mM *p*NPP in PBS was added to each well and incubated, the activity of alkaline phosphatase was then terminated by addition of an aqueous 1 M NaOH (15 μl) to each well. The absorbance of each well was determined at 405 nm using a

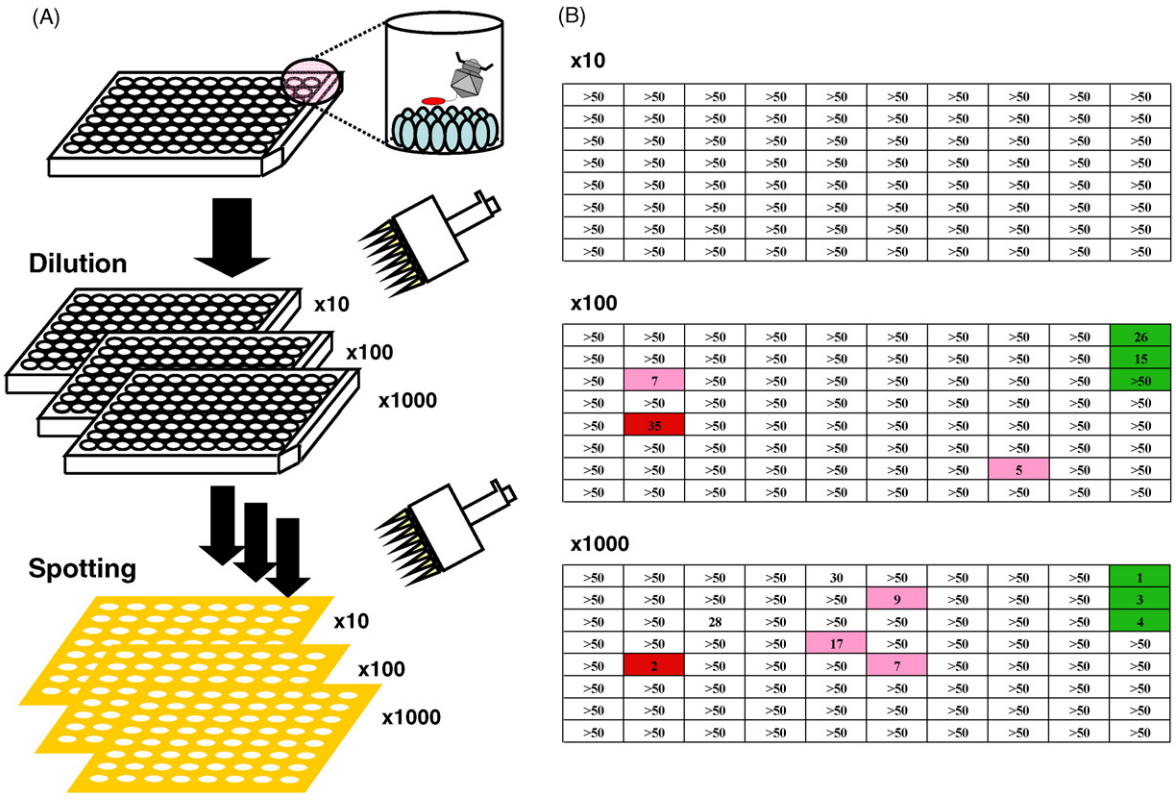

Fig. 4 – Schematic diagram and results of multiwell plate assay based on T7 phage display. (A) When T7 phage particles expressing $p53_{12–36}$ interacted with GST–$MDM2_{25–117}$ immobilized plates, nutlin 3 or compounds from the natural product library were added into each well. After a short incubation to allow protein–protein interaction, the wells were washed. Bound phage particles were recovered in 200 μl 1% SDS. The recovered phage particles from each well were diluted to 10, 100 and 1000 times in PBS of multiwell plates by using multi-channel pippeter. Each 5 μl diluted recovered phage solution was spotted onto a lawn of growing *E. coli* to count the plaques formed on each square plate by using multi-channel pippeter. (B) Three 96-well plates are illustrated as boxes containing small rectangular boxes to represent wells. After dilution and spotting, the numbers of the bound phage particles in each well was determined by PFU assay as the number of plaques formed. Colors in small boxes indicate the presence of less recovered phage particles by the inhibition of interaction of GST–$MDM2_{25–117}$ immobilized on wells and $p53_{12–36}$ expressed on T7 phage particles. Colored small boxes in green, red and pink indicate addition of nutlin 3, hit compounds such as dehydroaltenusin from the natural product library and false positive compounds, respectively. False positives were identified using the single clone check for recovered phage particles.

high concentrations (1 and 10 mM) of SDS. However, no detectable loss of GST–$MDM2_{25–117}$ protein occurred after treatment with the concentrations of SDS (1–100 μM). This result suggested that the immobilized GST–MDM2 was not washed with the treatment by the concentrations of SDS. Furthermore, GST–$MDM2_{25–117}$ protein did not elute from the immobilized wells after addition of nutlin 3 (10 nM to 10 μM) (Table 1). Since the concentrations of SDS (1–100 μM) and nutlin 3 (10 nM to 10 μM) did not elute GST–$MDM2_{25–117}$ protein from plate, our natural product library was screened using a concentration of 100 μM for each compound.

Phage particles displaying $p53_{12–36}$ were added to immobilized GST–$MDM2_{25–117}$ protein in a 96-well plate format. Each solution of natural product was added to individual wells using a multi-channel pipette, allowing rapid screening for inhibitors from a natural product library. After addition of the test compound (100 μM), the number of eluted phage particles from each well was determined by plaque assay. A solution of nutlin 3 at a final concentration of 10 μM was used as a positive control in this screening procedure. These experiments confirmed that the number of recovered phage particles decreased upon addition of nutlin 3. Our results indicate that the addition of inhibitor successfully interfered with the interaction between immobilized GST–$MDM2_{25–117}$ and $p53_{12–36}$ displayed on T7 phage particles. We then applied this facile assay on a natural product library to screen inhibitors of the interaction and tested whether 70 natural products inhibited the interaction of GST–$MDM2_{25–117}$ and $p53_{12–36}$ displayed on T7 phage particles.

Multiwell plate assay using a 96-well plate format resulted in the discovery of a single candidate inhibitor, dehydroaltenusin (Fig. 4B and Fig. 5A). Further analyses confirmed dehydroaltenusin to be an inhibitor of the interaction between GST–$MDM2_{25–117}$ and $p53_{12–36}$ displayed on T7 phage particle,

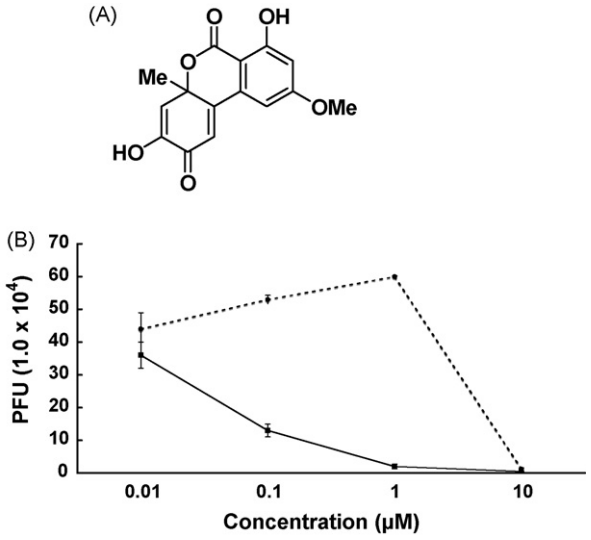

Fig. 5 – The structure and inhibition assay for dehydroaltenusin. (A) The structure of dehydroaltenusin and (B) the numbers of PFU (1.0×10^4) of the recovered phage particles were determined and expressed on the Y-axis. The X-axis shows the concentration of nutlin 3 and dehydroaltenusin. Closed boxes on the straight line and closed circles on the dotted line indicate the PFU of recovered phage particles in the presence of nutlin 3 or dehydroaltenusin from GST–MDM2$_{25–117}$ immobilized wells, respectively. All data shown here were determined by mean and standard deviations of three replicated assays to confirm reproducibility.

although the activity was 100-fold weaker than that of nutlin 3 (Fig. 5B). As shown in Table 1, immobilized GST–MDM2$_{25–117}$ was not displaced from the well by addition of a solution of dehydroaltenusin (100 μM). These results suggested that dehydroaltenusin did not simply elute the immobilized GST–MDM2$_{25–117}$ from the plates but inhibited the interaction of GST–MDM2$_{25–117}$ and p53$_{12–36}$ displayed on T7 phage particles. Thus, our novel screening procedure based on T7 phage display suggested that dehydroaltenusin was identified as a candidate inhibitor of the interaction between MDM2 and p53.

4. Discussion

It is currently of great interest to analyze protein–protein interactions, particularly with regard to those that regulate cellular events [1–4]. Specific inhibitors of these interactions are extremely useful both as a biological research tool and as a potential therapeutic agent [6–10]. Inhibitors of protein–protein interactions are usually identified either by screening natural products or by computer-aided molecular design based on structural studies. Both techniques require an assay system, preferably one that is convenient and can be performed at high throughput.

In this study, we aimed to establish a facile multiwell plate assay system for the identification of candidate inhibitors, based on the T7 phage display method. We initially analyzed the well-characterized interaction of p53 with MDM2 using the inhibitor nutlin 3 to construct a model assay system [5,6]. The p53–MDM2 interaction was performed on T7 phage particles (Figs. 1–3). The interaction and inhibition tests were performed in half a day, which is considerably quicker than the yeast two-hybrid system. In detail, it took approximately 6–7 h, including pipetting manipulation, to screen inhibitors in 96-well plate assays. Even though phage display is a common method to screen ligand-binding peptides from a library displayed on phage particles, the phage display described here is a method to screen peptide-binding ligands from a small-molecule library. In addition, the assay does not require any expensive equipment, such as SPR and FCS. This facile multiwell-plate assay was achieved by using multi well plates and multi channel pipettes (Fig. 4). Since nutlin 3 precisely inhibited the MDM2–p53 interaction as a positive control in triplicate assays, the screening represented here was a highly reproducible and reliable method. The reproducibility and reliability were also observed when this method picked dehydroaltenusin to be an inhibitor in three times repeated triplicate assays.

Our screening identified dehydroaltenusin as an inhibitor for the interaction of the immobilized GST–MDM2$_{25–117}$ and p53$_{12–36}$ displayed on T7 phage particles along with well-known inhibitors nutilin 3 and anti p53 antibody. Since nutilin 3 and anti-p53 antibody also inhibited the interaction between full length p53 and full length MDM2 as nutlin 3, dehydroaltenusin can also be a candidate inhibitor between full length p53 and full length MDM2 as nutlin 3. Both dehyroaltenusin and nutlin 3 are potential anticancer agents [9]. Furthermore, dehydroaltenusin has been shown to inhibit the proliferation of cancer cells [20,21]. For these reasons, dehydroaltenusin could be of interest as a candidate lead compound for an anticancer drug. However, dehydroaltenusin is also an inhibitor of DNA polymerase α and myosin light chain kinase [20–23]. In addition, dehydroaltenusin is a 100-fold weaker inhibitor for the interaction of GST–MDM2$_{25–117}$ and p53$_{12–36}$ displayed on T7 phage particles than nutlin 3 (Fig. 5B). Thus, further development of dehydroaltenusin is required in order to generate a viable anticancer agent. Additional experiments are also required to ascertain whether dehydroaltenusin has the same mode of action as nutlin 3 and is an in vivo inhibitor between full length p53 and full length MDM2.

In the present study, we established a novel drug discovery method to screen for candidate inhibitors of protein–protein interactions using facile phage display. We propose to call this methodology ‘reverse phage display’.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research (The Ministry of Education, Culture, Sports, Science and Technology of Japan, Japan Society for the Promotion of Science) and by Academic Frontiers Research Promotion Program (The Ministry of Education, Culture, Sports, Science and Technology of Japan).

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