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Analysis of the Resveratrol-binding Protein using Phage-displayed Random Peptide Library

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Abstract Resveratrol, a plant polyphenol, is found in significant amounts in the skin of grapes and in some traditional herbs. It is reported to exert different biological activities, such as inhibiting lipid peroxidation, scavenging free radicals, inhibiting platelet aggregation, and anticancer activity. In order to screen the resveratrol-binding proteins, we synthesized biotinylated resveratrol, purified by liquid chromatography and immobilized it into streptavidin-coated microplate wells. 3-(4,5-Demethylthiazol-)-2,5-diphenyl tetrazolium bromide assay showed little change in the anticancer activity of biotinylated resveratrol *in vitro*. A random library of phage-displayed peptides was screened for binding to immobilized resveratrol to isolate resveratrol-binding proteins. Several peptides were found to bind to resveratrol specifically, which was proven by enzyme-linked immunosorbent assay. Through amino acid sequence analysis of the selected peptides and human proteins using the BLAST program, the results showed that resveratrol has an affinity for various proteins such as breast cancer-associated antigen, breast cancer resistance protein, death-associated transcription factor, and human cyclin-dependent kinase. These results demonstrate that our study provides a feasible method for the study of binding proteins of natural compounds using a phage-displayed random peptide library.

Key words resveratrol; binding protein; phage display; anticancer activity

Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin, a natural polyphenol that has been found in a variety of dietary and medicinal plants including grapes and the root of *Polygonum cuspidatum.* The latter has traditionally been used in China for the treatment of inflammation, hepatitis and osteomyelitis [1,2]. Resveratrol was first detected from grapevines in 1976 by Langcake and Pryce [3], who found that this compound was synthesized by leaf tissues in response to fungal infection (mainly *Botrytis cinerea*) or exposure to ultraviolet light. Because of its high concentration in grape skin, a significant amount of resveratrol is present in wine, especially red wine. It has been suggested that resveratrol might be partially responsible for the beneficial effect of red wine in protecting against coronary heart disease (e.g. the French paradox) [4,5]. However, the possible application of resveratrol in cancer treatment has recently been proposed. Resveratrol was demonstrated to have chemopreventive effects in different systems based on its remarkable inhibition of diverse cellular events associated with three major cancer stages, tumor initiation, promotion and progression [6]. Resveratrol has also been shown to inhibit the growth of a number of human cancer cell lines *in vitro*, including human breast cancer cell lines MCF-7 and MDA-MB-231 [7], human liver cancer cell line Hep G2 [8], and human prostate cancer cell lines DU-145, PC-3 and JCA-1 [9]. Resveratrol could inhibit the growth of breast cancer cells in spite of estrogen receptor (ER)-positive (MCF-7) or ER-negative (MCF-10, MDA-MB-231). It has been suggested that resveratrol has several important biological functions, such as inhibition of protein kinase C, D and protein kinase (CKII) activity [10–12], and modulation of human mammary epithelial cell *O*-acetyltransferase, sulfotransferase, and kinase activation of the heterocyclic amine carcinogen *N*-hydroxy-PhIP [13], and others. However, the molecular

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mechanism of anticarcinogenesis of resveratrol is still unknown.

Methods to identify proteins that interact with a specific ligand are very limited. Among them, the phage-displayed random peptide library is a relatively successful molecular tool for investigating novel peptides that bind to a target. In this library the dodecapeptides are displayed on the surface of bacteriophage M13 [14,15]. Such phages thereby become vehicles for expression that not only carry the nucleotide sequence encoding expressed proteins, but also have the capacity to replicate [16]. Peptides with high affinity and specificity for a target molecule can be identified and isolated through multiple rounds of phage-based selection for binding to a target molecule. Such phage libraries have been used successfully to identify proteins that specifically bind to immobilized doxorubicin [17].

Because of the extraordinarily high affinity of the binding interaction of biotin to streptavidin, the key step is to make a biotinylated compound (bifunctional molecules), which contains both a biotin and a substrate unit for phage display screening [18], without changing its original bioactivity.

The objective of this work was to identify potential resveratrol targets using phage display technology. We synthesized a novel compound, biotinylated resveratrol, and compared its anti-tumor effects on human breast cancer cell line MCF-7 with natural resveratrol. Phage clones were then selectively amplified during the biopanning procedure using a phage display library. Finally we determined the amino acid sequences for the peptides selected, and compared these with the protein sequences available from the BLAST databases. We hope that these findings have valuable implications of the anti-tumor activities of resveratrol.

Materials and Methods

Reagents

Resveratrol and 3-(4,5-demethylthiazol-)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, USA). The Ph.D.-12 Phage Display Peptide Library Kit (#E8110SC; New England Biolabs, Beverly, USA) contained 1.5×10^{13} plaque-forming units (pfu) per milliliter with a complexity of 2×10^{9} independent peptide sequences. All other chemicals and solvents were of analytical grade.

Preparation of biotinylated resveratrol

Biotinylated resveratrol was synthesized in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine as catalyst. Resveratrol (744 mg, 3.26 M) was mixed with DCC (168 mg, 0.815 M) and 4-dimethylaminopyridine (10 mg, 0.08 M) in dry N,N-dimethylformamide (DMF; 10 ml). Biotin in DMF (199 mg, 0.815 M) was slowly added to this mixture in a dropwise manner. This solution was stirred for 24 h at room temperature. In order to obtain and purify the target compound, the reaction mixture was separated by a chromatographic column (Resource RPC 100 ml; Amersham Pharmacia Biotech, Uppsala, Sweden) connected with a fully automated liquid chromatography system (AKTA Explorer 100; Amersham Pharmacia Biotech), which was designed for method development and research applications. We selected acetonitrile and water for the mobile phase at a flow rate of 10 ml/min. Finally, the freeze-dried biotinylated resveratrol was prepared and identified by reversed phase-high performance liquid chromatograph-mass spectrometry (RP-HPLC-MS) (Platform ZMD 4000; Waters, Milford, USA) [19].

Cell culture

The human breast epithelial cell line MCF-7, an estrogen receptor-positive cell line derived from an *in situ* carcinoma, and human liver cancer cell line Hep G2 were obtained from ATCC (Drive Rockville, USA). RPMI 1640 medium (Gibco, Grand Island, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 2 mM *L*-glutamine, 100 U/ml penicillin and streptomycin (Gibco), 0.4% trypsin was used for cell culture as the basal medium. MCF-7 was routinely cultured with the above culture medium at 37 ºC in a humidified atmosphere of 5% CO_2.

MTT assay for cell viability

For treatment with drugs, resveratrol and biotinylated resveratrol were dissolved in culture medium containing 0.1% dimethylsulfoxide (DMSO) to obtain a 400 μM stock solution, and then diluted with culture medium to obtain the working solution. Untreated control cells were incubated under identical conditions with the same volume of culture medium containing 0.1% DMSO. Cell viability was assessed by MTT assay [20]. Briefly, cells were counted and plated at the same initial density into a 96-well plate with 8000 cells per well in 100 μl medium. After 24 h incubation, the old medium was removed and the fresh medium containing drugs of different concentrations was added to the wells, which was further incubated for 48 h. Then 20 μl MTT solution (5 mg/ml) was added to each well and, after 4 h incubation, the medium containing MTT was replaced with 150 μl DMSO. The plate was further incubated for 15 min at 37 ºC in the dark. Finally we

measured the absorbance (A_{570}) of each well on a microplate reader (Multiskan MK3; Thermo Labsystems, Marietta, USA). All experiments were performed three times.

Immobilization of biotinylated resveratrol and biopanning experiments

The biotinylated resveratrol was dissolved in $NaHCO_3$ buffer (0.1 M, pH 8.6) and transferred to a well of the streptavidin-coated plate. After incubation for 12 h at 4 ºC, the well was washed six times with TBST [0.1% (*V*/*V*) Tween-20 solution in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl)]. The Ph.D.-12 phage-displayed random peptide library (1×10^{11} pfu) in 100 μl of TBS buffer was added to the resveratrol-immobilized plate, and the plate was shaken gently for 1 h at room temperature. To remove the unbound phages the plate was washed 10 times with TBST, and the phages, which had adsorbed resveratrol, were eluted by 100 μl of 0.2 M glycine-HCl (pH 2.2). The elution was neutralized to pH 7.5 with 15 μl Tris-HCl (pH 9.1) immediately. The bound phages were amplified using *Escherichia coli* ER2738 to make enough copies for the next round of biopanning. After five rounds of biopanning (the concentration of Tween-20 in the washing solution used in the second, third, fourth and fifth eluting rounds was increased to 0.3%, 0.5%, 0.5% and 0.5%, respectively), the bound phages were eluted and plated on Luria Broth agar plates containing isopropyl β-*D*-thiogalactopyranosid and X-gal to prevent contamination. The blue monoclone was picked and amplified to sequence its DNA. The sequencing primers were M_{13} U: 5′-GTTCCTTTCTATTCTCACTC-3′ and M_{13} L: 5′-TCGTCACCAGTACAAACTAC-3′. Amino acid sequence comparisons with all available human protein sequences were performed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

ELISA binding assay

To test whether or not the four selected monophages could bind to biotinylated resveratrol specifically, ELISA assays were carried out. The plates were coated with streptavidin for 24 h at 4 ºC, blocked with bovine serum albumin, and washed six times with 0.5% PBST [0.5% (*V*/*V*) Tween-20 solution in phosphate-buffered saline buffer]. Biotinylated resveratrol was added and allowed to bind for 24 h at 4 ºC. After washing with 0.5% PBST, 10^{10} amplified monophages were added, incubated for 2 h at 37 ºC. After washing, horseradish peroxidase-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) was added, incubated for 2 h at 37 ºC. ABTS (Amresco, Cleveland, USA) was used in color-development. Absorbance at 405 nm (A_{405}) of each well was read on a microplate reader (Multiskan MK3, Thermo Labsystems). Wells coated with streptavidin or streptavidin-biotin conjugate were used as negative controls.

Results

Characterization of biotinylated resveratrol

In this study, for the analysis and identification of biotinylated resveratrol, we developed a method by validated RP-HPLC system with diode array and ion-trap mass spectrometric detection. RP-HPLC-MS, using electrospray ionization with 25% ammonia solution as the sheath liquid, was performed in the selected ion monitoring mode at *m*/*z* 453 $[M-H]^-$. It is well known that MS detection is highly linear for all investigated analytes including all kinds of reagent, catalyst and production, and the limits of detection were in the low nanogram range.

The RP-HPLC conditions were as follows: chromatographic column [SunFire C_{18}, 5 μm, 2.1 mm×150 mm (Part No. 186002541, Lot No. 0108143521, Waters, Milford, USA)]; Eluent A was 15% methanol+1% acetic acid, Eluent B was 85% methanol+1% acetic acid and Eluent C was pure methanol; the elution gradient was that 90% Eluent A+10% Eluent B to 0% Eluent A+100% Eluent B in 20 min, to 100% Eluent C at 5 min; flow-rate 0.3 ml/min; temperature 30 ºC; injection volume 10 μl.

Fig. 1 shows a typical MS spectrum of biotinylated resveratrol in the negative ion mode. A molecular ion corresponding to the most intense peak was observed at *m*/*z* 227.8 and corresponded to resveratrol. The $[M-H]^-$ peak at *m*/*z* 453.9 was assigned to biotinylated resveratrol. In positive mode, the RP-HPLC-MS experiments also gave a similar spectrum (not shown) with an *m*/*z* value of 455.6 for the biotinylated resveratrol. Altogether, the results indicated that our designed chemical synthesis with catalyst led to the formation of biotinylated resveratrol *in vitro* with a molecular weight of 454 Da, as expected.

Anti-tumor effects of biotinylated resveratrol on cancer cell growth *in vitro*

Resveratrol has also been shown to possess strong anti-tumor activity against several cancer cell lines *in vitro*, including MCF-7 [7] and Hep G2 [8], at micromolar concentrations. To evaluate the bioactivity of biotinylated resveratrol compared to natural resveratrol, MCF-7 and Hep G2 cells were cultured in the presence of biotinylated

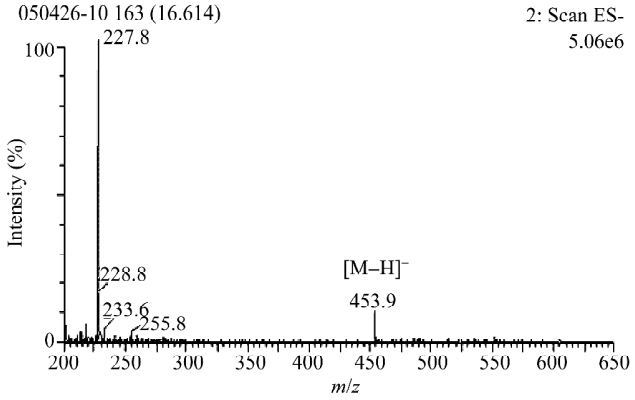

Fig. 1 Mass spectrometry of biotinylated resveratrol in negative ion mode

Capillary voltage (EIS^-) was 3.88 kV; cone voltage was 30 V; nebulization temperature was 120 ºC; expansion region temperature was 300 ºC; helium flow-rate was 4.2 liters per hour.

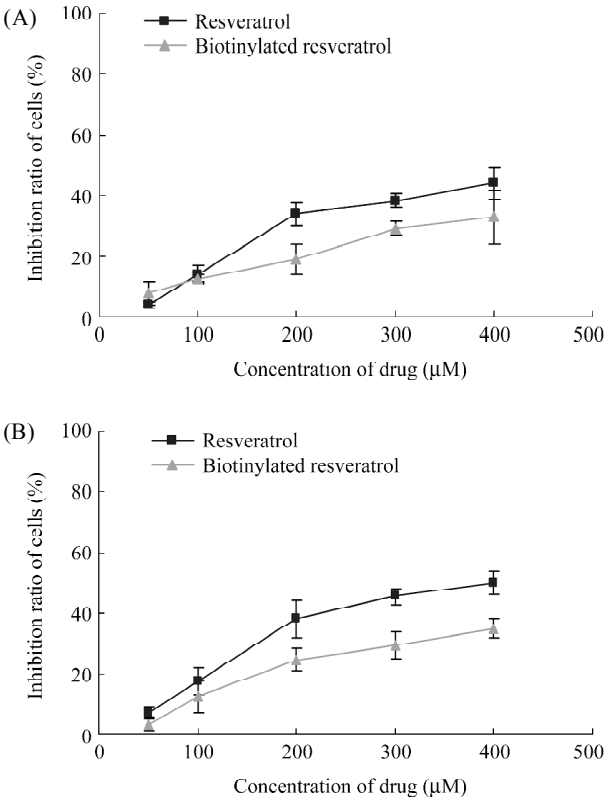

Fig. 2 Biotinylated resveratrol and natural resveratrol decrease cell viability in human liver cancer cells Hep G2 (A) and human breast cancer cell line MCF-7 (B)

Cells were treated with different concentrations of drugs and viability was determined by the 3-(4,5-demethylthiazol-)-2,5-diphenyl tetrazolium bromide reduction assay. Measurements were taken in duplicate in three independent experiments.

resveratrol or natural resveratrol. The bioactivity was determined with a growth inhibition ratio of cells after 48 h culture.

The results, shown in **Fig. 2**, illustrate that more than 30% cell growth was inhibited in the two cell lines exposed to 300 μM biotinylated resveratrol, and 40% growth inhibition ratio on resveratrol in the same conditions. We also found the inhibition of the two reagents was dose-dependent in both MCF-7 and Hep G2 cells. As shown in **Fig. 2(A)**, when Hep G2 cells were treated with biotinylated resveratrol at a low concentration (50 μM), the cells propagated without influence. However, as the concentration of biotinylated resveratrol increased, the number of cells decreased sharply. The same phenomenon is shown in MCF-7 cells in **Fig. 2(B)**, suggesting the same sensitivity to biotinylated resveratrol in both cell types. Because of the low concentration of biotinylated resveratrol in medium (400 μM), it has an inhibition ratio of 34.68% and 32.95% in MCF-7 and Hep G2 cells, respectively. The MTT results suggested that resveratrol and biotinylated resveratrol could inhibit the cell viability of MCF-7 and Hep G2 [21].

Based on the results above, we consider that although the anti-tumor activity and solubility of biotinylated resveratrol decreased to some degree, this effect was so limited that we predict that resveratrol linked with biotin changed its anti-tumor activity *in vitro* only a little.

Resveratrol-specific peptide biopanning by phage-displayed random peptide library

The phage-displayed random peptide library that we use was pooled to contain a diversity of 2×10^9 independent and different peptide sequences using a previously reported design. The M_{13} phage particles whose recombinant pIII proteins might bind to resveratrol were isolated by biopanning as detailed in "Materials and Methods" using a derivatized resveratrol with a biotin group and immobilized on a streptavidin-coated plate. Five pIII structural proteins present at the tip of the virion each possessed a random 12 amino acids extension at their amino terminus, coded by a random synthetic oligonucleotide inserted into the corresponding position in the gene for pIII. The sequences of inserted oligonucleotides were determined and translated to obtain the sequence of the displayed peptide [16,22]. The M_{13} phage infects *E. coli* ER2738 and replicates without lysis of the host during the procedure of the selected phage's amplification. The titer of eluted phages and their apparent affinity increased with each round despite the use of increasingly stringent wash conditions (**Fig. 3**). The phage titer of the eluted solutions increased

from 6.1×10^2 pfu after the first round to 1.2×10^4 pfu at the fifth round of binding and elution. In **Fig. 3**, we found that the titer of eluted phages fell off sharply when the concentration of Tween-20 in the washing solution was increased from 0.3% to 0.5%. Note that 0.5% Tween-20 can wash away the majority of inferior affinity phages binding to resveratrol more efficiently than 0.1% and 0.2% Tween-20 while superior affinity phages remain. So we performed the fourth and fifth biopanning rounds until we obtained a constant yield of eluted phages. These results indicated that phages binding to resveratrol were selectively isolated and amplified by the biopanning procedure.

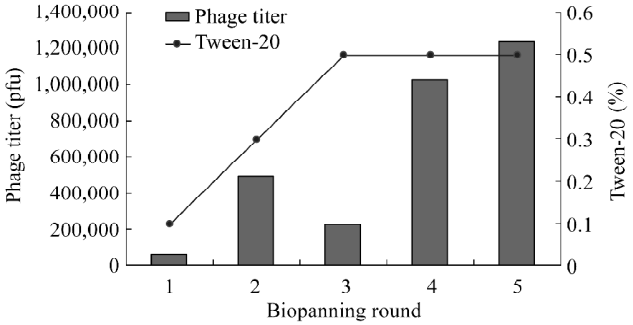

Fig. 3 Phage titer was determined for each eluted solution in each round

After five rounds of biopanning, 20 eluted phages plated on Luria Broth agar plates containing isopropyl β-*D*-thiogalactopyranosid and X-gal were randomly chosen to be amplified for extracting single-stranded DNA of the M_{13} phage, which was used as the polymerase chain reaction template. Then we finally obtained the polymerase chain reaction products of approximately 200–300 bp. The sequencing primers were M_{13} U: 5′-GTTCCTTTCTATTCTCACTC-3′ and M_{13} L: 5′-TCGTCACCAGTACAAACTAC-3′. Amino acid sequence comparisons were carried out using the BLAST program and all available human protein sequences. The results (**Table 1**) indicated that the primary structure analysis did not reveal any homology for four peptides exposed on the surface of the selected phages. As shown in **Fig. 4**, absorbance at 405 nm (A_{405}) of the well coated with streptavidin-biotinylated resveratrol conjugate was much higher than the other two, which meant that all four selected phage monoclones could bind to biotinylated resveratrol specifically.

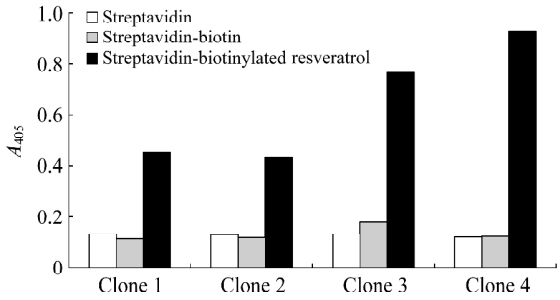

Fig. 4 Four selected phage monoclones binding to different antigens by enzyme-linked immunosorbent assay (ELISA)

The assay was carried out using a plate coated with streptavidin, streptavidin-biotin conjugate and streptavidin-biotinylated resveratrol conjugate, respectively.

Discussion

Resveratrol, also known as 3,5,4′-trihydroxystilbene, is a phytoalexin, a polyphenol used by plants to defend themselves from fungal and other forms of aggression. It is found in grape skin and red wine in substantial amounts [23]. As a plant polyphenol, resveratrol is an antioxidant and a free radical scavenger, and it has therefore been suspected to be responsible for the cardioprotective effects of red wine, usually described as the "French paradox" [24,25]. As the molecular mechanisms of the anticancer and chemopreventive effect of resveratrol are unknown,

Table 1 Sequence analysis of the resveratrol binding peptides

Peptide name	Peptide sequence	Occurrence in sequenced plaques†
Clone 1	Ser-Val-Ser-Val-Gly-Met-Lys-Pro-Ser-Pro-Arg-Pro	11
Clone 2	Trp-His-Trp-Ser-Trp-Gln-Arg-Asn-Tyr-Pro-Ser-Tyr	7
Clone 3	His-Thr-Pro-Leu-Gln-Thr-Pro-Val-Leu-Tyr-Pro-Lys	1
Clone 4	His-Leu-Lys-Val-Pro-Ser-Phe-Pro-Lys-Ser-Thr-Trp	1

† The frequency at which each peptide appeared in the group of sequenced plaques.

it is worth examining the resveratrol-binding protein.

It is well known that the interreaction of the properties of proteins or polypeptide is an important ramification in many areas of biology, ranging from medicine, to chemistry, to food. The use of the phage-displayed random peptide library is a popular approach to achieving this goal, because of its convenient amplification and evaluation. This technology is based on diverse answers to the same question: What is the binding target? This phage-displayed system requires molecules, which can be captured by a solid phase after reaction or which contain chiral substrates tethered to a solid phase. The immobilization of the drug is the first key step in the experiment. In this study we chose the biotin-streptavidin system because of the extraordinarily high affinity of the binding interaction of biotin to streptavidin, which is commonly used in phage display biopanning experiments. We had already synthesized a novel compound, biotinylated resveratrol. The reactants (biotin and excess resveratrol) brought about the single substitution reaction of esterification at room temperature, using DCC and DAMP as catalysts. By RP-HPLC coupled with electrospray ionization MS, biotinylated resveratrol (molecular weight 454 Da) was obtained and identified (**Fig. 1**). As a result, resveratrol could be immobilized on a solid support (streptavidin-coated plate) by non-covalent binding of the biotin-streptavidin system for phage display panning experiments. **Fig. 2** shows that biotinylated resveratrol could inhibit the proliferation of MCF-7 and Hep G2 cells in a dose-dependent manner, the same as the results for resveratrol using MTT assay. It is considered that the virgin spatial configuration of resveratrol binding a biotin group underwent little change, and its anticancer activity remained the same, although its solubility was lower. These results also mean that biotinylated resveratrol could provide the same binding site or binding spatial structure as natural resveratrol to bind the target protein after immobilization, which would only marginally affect the reliability of the biopanning experiment results.

Using a phage library displaying random peptides of 12 amino acids on its surface, four peptides were found that bind to resveratrol. No obvious homology was found for these peptides after five rounds of biopanning (**Fig. 3**; **Table 1**). Results from ELISA assay (**Fig. 4**) confirmed that the affinity between the four peptides and resveratrol was specific. In order to find out the potential target proteins of resveratrol, the above four selected resveratrol-binding peptides were compared with all human protein sequences available from the BLAST databases. Sequences with at least five residues coinciding with those of a peptide were selected. The results from sequence comparison revealed high homology with diverse proteins, including death-associated transcription factor, immunoglobulin E, F-box and leucine-rich repeat protein, protein kinase C and CKII, retinoblastoma-binding protein, breast cancer-associated antigen BRCAA1, oncostatin M receptor, polymerase (DNA directed), GTPase activating protein, UDP (uridine diphosphate)-glucuronosyltransferase, G-protein coupled receptor, breast cancer resistance protein (BCRP), voltage dependent t-type calcium channel alpha-1H subunit, transcription factor-like protein, purine nucleoside phosphorylase, human cyclin-dependent kinase 2, activating transcription factor, and zinc finger protein. According to published reports, some of these proteins have been identified as relevant to the anticancer activity of resveratrol. For example, protein kinase CKII is involved in cell proliferation and oncogenesis [25], and resveratrol was shown to inhibit the phosphotransferase activity of CKII. Studies revealed that resveratrol acted as a competitive inhibitor with respect to the substrate ATP and inhibited the catalytic reaction of CKII with guanosine triphosphate as substrate. These results suggested that resveratrol was likely to function by inhibiting oncogenic disease, at least in part, through the inhibition of CKII activity [12].

We also found that resveratrol can inhibit the viability of both MCF-7 and MCF-7/ADM cells induced to resistance with ADM by the gradually increasing concentrate method (resistant index 200 multiple) in a dose- and time-dependent manner *in vitro*. Flow cytometry showed that resveratrol induces the G_1 phase accumulation in MCF-7/ADM cells (data not shown). In this study, we tested the high affinity of the binding interaction of resveratrol to BCRP. BCRP is a more recently discovered multidrug ATP-binding cassette transporter, a member of the ATP-binding cassette gene "half-transporter" subfamily [27]. It has a drug resistance profile similar though not identical to P-glycoprotein and, like P-glycoprotein, is located at barrier sites [28] where it might influence entry of xenobiotic material. In recent years some published work has reported that many plant polyphenols interact directly with BCRP, including modulating its transport function and ATPase activity [29]. So it could be implied that resveratrol is directly transported by BCRP, resulting in possible conformational alterations to BCRP or effects on the cellular function of BCRP. This might explain why resveratrol inhibits the viability of MCF-7/ADM cells. However, this rational conjecture demands more powerful proof, such as ELISA assay and iRNA technology. The BLAST results also provided valuable information about the bioactivity of resveratrol, which includes not only antitumor, but also anti-inflammatory,

immunomodulating, and antiviral activities.

Our study provides an example of a feasible method of investigating the binding proteins of natural products, compounds or drugs. We made use of biotinylation, which did not significantly alter the activities of the original molecules, and a phage-displayed random peptide library.

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