THE JOURNAL OF
ANTIBIOTICS

Identification of Antibiotic Clarithromycin Binding Peptide Displayed by T7 Phage Particles

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Abstract Peptide libraries displayed by T7 phage, which contain random cDNA fragments insets, were screened for their ability to bind to a biotinylated derivative of clarithromycin. Phage particles bound to an immobilized derivative of the antibiotic were isolated and the inserted cDNA was amplified and sequenced. A common selected peptide sequence, composed of 19 amino acids, was obtained and a synthetic peptide with this sequence was produced. Surface plasmon resonance experiments showed that the synthetic peptide immobilized on a sensor chip bound to clarithromycin and the dissociation constant was determined to be 2.1×10^{-3} M. The dissociation constants of other macrolide antibiotics, erythromycin, roxithromycin, azithromycin and josamycin were also determined to be 5.4×10^{-3} M, 6.2×10^{-5} M, 1.1 M and 3.4×10^{-2} M, respectively. These results indicated that T7 phage display method might be useful to determine relatively weak interactions between small molecule drugs and the selected peptides which could represent a possible binding site conserved in binding proteins.

Keywords phage display, macrolide antibiotics, clarithromycin, surface plasmon resonance

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Introduction

It is currently of great interest to identify proteins that interact with small molecules, which will help us understand mammalian biochemical processes and possibly accelerate the development of novel pharmaceutical and agricultural agents [1~3]. One of the latest methods to identify small molecule-binding proteins is "phage display" reported by Smith and Petrenko [4, 5]. For example, affinity selection of a random library of peptides displayed on M13 bacteriophage was performed to identify paclitaxel (Taxol)-binding anti-apoptotic human protein Bcl-2, which has no significant sequence homology to that of tubulin [6]. Another interesting example used a T7 phage library of human liver cDNA to show that the anthracyclin antibiotic doxorubicin (brand name: Adriamycin), a widely used antineoplastic agent for solid tumors, binds to a nucleolus phosphoprotein hNopp140 [7].

Clarithromycin (CLA; chemical name—6-*O*-methylery-thromycin, **1**) [8] is a macrolide antibiotic that is widely used in clinical medicine. Macrolide antibiotics such as CLA specifically bind to the 50S subunit of the bacterial ribosome thereby interfering with protein biosynthesis [9]. However, the identity of human proteins that bind CLA remains uncertain. If T7 phage library could display CLA-binding peptides, the amino acid sequences might be conserved in a domain of CLA-binding proteins. To identify CLA-binding amino acid sequences, we used two T7 phage libraries containing cDNA fragment inserts from either human leukocytes or Drosophilae which display exhibiting random peptides on the capsid protein. Phage clones expressing a common peptide were selectively

amplified after biopanning with a biotinylated derivative of CLA. Using this technique we have identified a 19 amino acid clarithromycin-binding peptide. Furthermore, the dissociation constants between the synthetic peptide and CLA (**1**), erythromycin (ERY, **2**), roxithromycin (RXM, **3**), azithromycin (AZM, **4**) or josamycin (JSM) were obtained from surface plasmon resonance analyses.

Experimental

Materials

ERY (**2**) was purchased from Sigma-Aldrich (St. Louis, MO). Other macrolide antibiotics, CLA (**1**), RXM (**3**), AZM (**4**) and JSM, were extracted from tablets using CH_2Cl_2 and then purified by silica gel column chromatography. The structure of each macrolide and synthetic derivative was checked by both NMR (AVANCE DRX-400 and 600, Bruker BioSpin, Rheinstettin, Germany) and ESI-MS (Esquire3000 plus, Bruker Daltonics, Bremen, Germany). Chemical shifts in the NMR data were expressed by δ ppm from TMS as an internal standard. All NMR and MS data were processed with XWIN-NMR and Data Analysis, respectively. MS of the recombinant protein was determined on a MALDI-TOFMS (Reflex IV, Bruker Daltonics). Surface plasmon resonance data was collected on a Biosensor BIAcore3000 instrument (Uppsala, Sweden). Research grade streptavidin sensor chips were purchased from BIAcore (Sweden). DNA sequences were analyzed using an ABI PRISM^R 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Unless stated otherwise, all other reagents were of analytical grade and were purchased from Junsei Ltd. (Tokyo, Japan).

Synthesis of Biotinylated CLA (6)

N-Carbobenxyloxy-β-L-alanine (90 mg, 0.4 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 100 mg, 0.52 mmol) and 4-dimethylamino pyridine (DMAP, 6 mg, 0.05 mmol) were added to a solution of CLA (**1**, 200 mg, 0.26 mmol) in dry pyridine (10 ml), and the mixture was stirred for 5 days at 50°C. The reaction mixture was poured into water and extracted with ethyl acetate. After being dried over anhydrous Mg_2SO_4, the filtrate was evaporated *in vacuo* to give a syrup and then subjected to silica gel column chromatography to yield **5** (55.3 mg, 0.058 mmol, 22.3%). A positive ESI-MS of **5** showed *m/z* 953.5 as an M+H ion of $C_{49}H_{81}N_2O_{16}$. NMR (CDCl$_3$); macrolide part, ^{1}H: δ 5.05 (1H, dd, J=2.32, 11.12 Hz, H-13), 3.74 (1H, d, J=7.28 Hz, H-3), 3.72 (1H, d, J=7.8 Hz, H-10), 3.61 (1H, d, J=6.56 Hz, H-5), 3.02

(3H, s, H-22), 2.97 (1H, m, H-9), 2.83 (1H, dq, J=7.28, 9.12 Hz, H-2), 2.58 (1H, m, H-8), 1.96 (1H, m, H-14b), 1.88 (1H, m, H-4), 1.68 (1H, m, H-7b), 1.61 (1H, m, H-7a), 1.50 (1H, m, H-14a), 1.41 (3H, s, H-18), 1.21 (3H, d, J=9.12 Hz, H-16), 1.13 (3H, d, J=7.24 Hz, H-19), 1.11 (3H, d, J=6.88 Hz, H-20), 1.10 (3H, s, H-21), 0.84 (3H, t, J=7.6 Hz, H-15), 0.84 (3H, d, J=7.6 Hz, H-17), ^{13}C: δ 221.1 (C-9), 175.1 (C-1), 80.8 (C-5), 78.2 (C-6 and C-3), 74.6 (C-13), 74.2 (C-12), 69.1 (C-11), 50.5 (C-22), 45.2 (C-8), 45.0 (C-2), 38.7 (C-7), 38.0 (C-4), 19.8 (C-18), 21.0 (C-14), 10.6 (C-15), 15.9 (C-16), 17.9 (C-19), 16.1 (C-21), 12.3 (C-20), 8.9 (C-17); cladinose part, ^{1}H: δ 4.79 (1H, dd, J=7.44, 10.68 Hz, H-2'), 4.60 (1H, d, J=7.44 Hz, H-1'), 3.50 (1H, m, H-5'), 2.61 (1H, m, H-3'), 2.15 (6H, s, H-7' and H-8'), 1.75 (1H, m, H-4'a), 1.24 (3H, d, J=6.04Hz, H-6'), 1.23 (1H, m, H-4'b), ^{13}C: δ 100.3 (C-1'), 71.5 (C-2'), 68.3 (C-5'), 63.8 (C-3'), 39.7 (C-7' and 8'), 28.22 (C-4'), 21.5 (C-6'); desosamine part, ^{1}H: δ 4.92 (1H, d, J=4.48 Hz, H-1''), 3.95 (1H, dq, J=6.32, 6.24 Hz, H-5''), 3.32 (3H, s, H-8''), 3.05 (1H, dd, J=8.88, 8.88 Hz, H-4''), 2.36 (1H, br s, H-2''a), 1.61 (1H, dd, J=4.52, 2.28 Hz, H-2''), 1.29 (3H, d, J=6.24 Hz, H-6''), 1.25 (3H, s, H-7''), ^{13}C: δ 95.9 (C-1''), 77.8 (C-4''), 72.9 (C-3''), 65.9 (C-5''), 49.4 (C-8''), 21.5 (C-7''), 18.6 (C-6'').

The solution of **5** (46 mg, 0.048 mmol) in ethanol (20 ml) was stirred for 2 hours at room temperature in the presence of 10% Pd/C (10 mg) under a hydrogen atmosphere. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. The residual oil (39 mg) and 6-(biotinamidocaproylamide)caproic acid *N*-hydroxy-succinimide ester (30 mg, 0.05 mmol) was dissolved in dry pyridine (10 ml) and stirred for 2 hours at room temperature. The reaction mixture was evaporated *in vacuo*, and the residue was subjected to silica gel column chromatography to give **6** (41.5 mg, 0.033 mmol, 69%). A positive ESI-MS of **6** showed *m/z* 1271.7 as an M+H ion of $C_{63}H_{111}N_6O_{18}S$.

Construction of T7 Phage Library from *D. melanogaster*

Poly(A)+ RNA, random primers, 5'-methylated dCTP, T4 DNA polymerase, *Eco*RI/*Hind*III linkers, *Eco*RI, *Hind*III, T7Select10-3b vector and T7 Packaging Extracts were purchased from Novagen (Madison, WI). Aliquots of 80 μg of total RNA were extracted from *D. melanogaster* Kc cells. Oligotex-dt30 〈super〉 (Takara, Kyoto, Japan) was used for a second round of isolation with minimal loss of material to produce poly(A)+ RNA suitable for random primed cDNA synthesis. cDNA synthesis was primed with 4 μg of poly(A)+ RNA with random primers, and then 5'-methylated dCTP was incorporated into both strands without extraction or precipitation between the first and

second strand synthesis. The cDNA were treated with T4 DNA polymerase to generate fragments with blunt ends and then ligated with directional *Eco*RI/*Hind*III linkers. Following linker ligation, the cDNA was digested sequentially with *Hind*III and *Eco*RI then inserted into *Eco*RI/*Hind*III digested T7Select10-3b vector arms. The resultant cDNA was inserted into the *Eco*RI/*Hind*III site of T7 phage 10-3B vector and packaged into phage. The titer of this library was 1.6×10^{10} pfu/ml.

Construction of T7 Phage Library from Human Leukocytes

The *Hind*III random primer (dTTNNNN), MMLV-RT, methylated dNTPs, *E. coli* DNA pol I, T4 DNA polymerase and *Eco*RI/*Hind*III linker were purchased from Novagen. The T7 phage library displaying alternative peptide sequence was made as follows. A 400 μg aliquot of human leukocyte total RNA, extracted by RNeasy Maxi (Qiagen, Amsterdam, The Netherlands), was applied to an oligotex-dT30 resin (Takara) to purify poly(A)$^+$ mRNA. We used 4 μg of poly(A)$^+$ mRNA primed with *Hind*III random primer (dTTNNNN) for first strand cDNA synthesis using MMLV-RT and methylated dNTPs. Second strand cDNA was synthesized with *E. coli* DNA pol I. The double stranded cDNA was made flush with T4 DNA polymerase. The blunt-ended double stranded cDNA was ligated with *Eco*RI/*Hind*III linker and digested with *Eco*RI and *Hind*III. The resultant cDNA was inserted into the *Eco*RI/*Hind*III site of T7 phage 10-3b vector and packaged into phage. The titer of the primary recombinants was 4×10^6 pfu/ml.

Biopanning and Sequence Analysis

Following four rounds of selection, almost 40 plaques were arbitrarily isolated from LB plates and each was dissolved in phage extraction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 6 mM MgSO$_4$). In order to disrupt the phage, the extract was heated to 65°C for 10 minutes. The phage DNA was then amplified by PCR, using the forward primer (5′-GACTTGGCTCTGGAGCGCGC-3′) and the reverse primer (5′-AACCCCTCAAGACCCGTTTA-3′). After amplification, the PCR fragments were purified with ExoSAP-IT (Amersham Biosciences, Uppsala, Sweden) and precipitated by adding 100% ethanol. The purified PCR fragments were then sequenced. Based on the sequence results, the amino acid sequence displayed on the T7 phage capsid was determined. Candidate clones were amplified and used to check the affinity to the biotinylated derivative of CLA (**6**).

Surface Plasmon Resonance (SPR)

Binding analysis between CLA and a synthetic peptide "NSRMRVRNATTYNS" (AnyGen, Kwang-ju, Korea) was performed using a Biosensor BIAcore instrument (BIAcore 3000, Biacore AB) with a CM5 research grade sensor chip (Biacore AB). The synthetic peptide (332 μg/ml, 170 μl) in coupling buffer (10 mM Na$_2$CO$_3$/NaHCO$_3$, pH 8.5) was injected over a CM5 sensor chip at a flow rate of 10 μl/minute to capture the peptide on the carboxymethyl dextran matrix of the chip by amine coupling at 25°C. The surface was activated by injecting a solution containing 0.2 M *N*-ethyl-*N*′-dimethylaminopropyl carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) for 14 minutes. The peptide was injected and the surface was then blocked by injecting 1 M ethanolamine at pH 8.5 for 14 minutes. This reaction immobilized about 4700 response units (RU) of the peptide. Binding analyses of macrolides were performed in running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20, 10% DMSO) at a flow rate of 20 μl/minute at 25°C. To measure the binding specificity and kinetics for the 19 amino acid peptides, an 80 μl solution of CLA (0, 125, 250 and 500 μM), ERY (1, 2 and 4 mM), RXM (0, 1, 2 mM), AZM (0, 1, 2, 4 mM) or JSM (0, 0.5, 1, 2 mM) was injected for 240 seconds. Association and dissociation were each measured for 120 seconds at 20 μl/minute. Kinetic parameters were determined using the software BIA evaluation 3.1 (Biacore AB).

Results

Synthesis of Biotinylated Clarithromycin

A derivative of CLA biotinylated at C2′ on the carbohydrate ring was prepared from CLA (**1**, Fig. 1A). There were three synthetic steps to give the biotinylated derivative (**6**) in 14% total chemical yield: *step* 1, the condensation reaction with CLA and *N*-benzyl-β-alanine to introduce a linker portion for biotin at C2′ *step* 2, reductive hydrogenation to remove the protective benzyl group *step* 3: biotinylation with 6-(biotinamidocaproylamido)caproic acid *N*-hydroxysuccinimide ester to yield biotinylated-CLA (**6**). The condensation reaction (*step* 1) gave three products that could be separated chromatographically. NMR was used to determine the structure of each compound. The three products were: a disubstituted compound at C2′ on cladinose and C2″ on desosamine, and two mono substituted products at C2′ or C2″. In the NMR data of the mono substituted products, the ^{1}H chemical shift (2H, δ 3.2 ppm from TMS as an internal standard) at C2′ of CLA shifted to 4.79 (2H), whereas those at C2″ did not shift significantly (δ 1.61 and 2.36 for each proton signal). In the HMBC experiment, the ^{1}H signal at C2′ (δ 4.79) had a
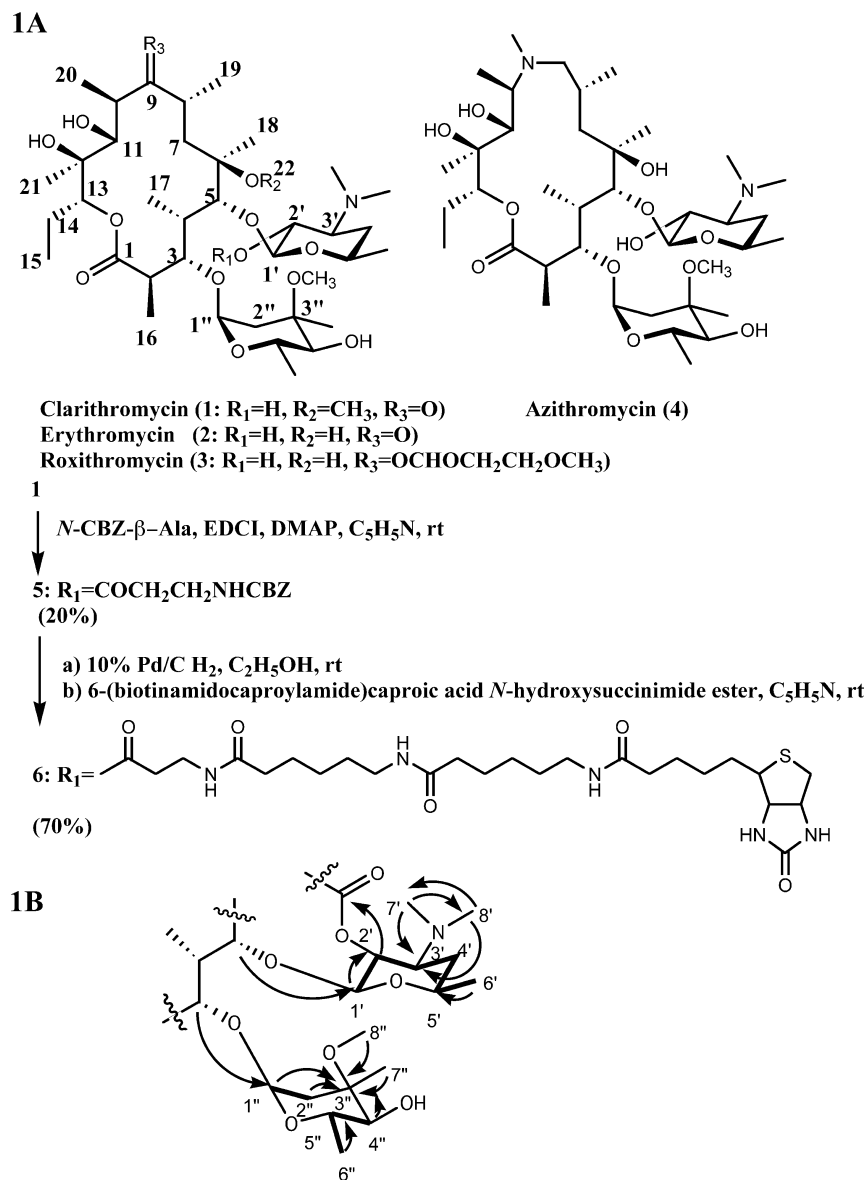
1A

Clarithromycin (1: R₁=H, R₂=CH₃, R₃=O)   Azithromycin (4)
Erythromycin   (2: R₁=H, R₂=H, R₃=O)
Roxithromycin (3: R₁=H, R₂=H, R₃=OCHOCH₂CH₂OCH₃)

1

N-CBZ-β–Ala, EDCI, DMAP, C₅H₅N, rt

5: R₁=COCH₂CH₂NHCBZ
(20%)

a) 10% Pd/C H₂, C₂H₅OH, rt
b) 6-(biotinamidocaproylamide)caproic acid N-hydroxysuccinimide ester, C₅H₅N, rt

6: R₁=

(70%)

1B

Fig. 1 A: Structures of clarithromycin (CLA, **1**), erythromycin (ERY, **2**), roxithromycin (RXM, **3**), azithromycin (AZM, **4**) and synthetic route to biotinylated-clarithromycin (**6**). There are three synthetic steps to yield **6**, *step* 1: the condensation reaction with CLA (**1**) and N-benzyl-β-alanine to give mono ester **5**, *step* 2: reductive hydrogenation, *step* 3: biotinylation with 6-(biotinamidocaproylamido)caproic acid N-hydroxysuccinimide ester. See text for details. B: Partial identification by HMBC and COSY. 1H-1H Correlations in the COSY and HMBC of N-carbobenxyloxy-β-L-alaninyl clarithromycin are expressed as thick lines and arrows, respectively.

cross peak with an ester carbon signal at δ 170.4, implying the presence of a C2′ ester of N-benzyl-β-alanine. Because the C2″ derivative gave a complex mixture of products upon hydrogenation, the mono substituted product at C2′ was used as the starting material for the subsequent reactions.

Selection of High Affinity Phage by Biopanning

For selection by biopanning, the T7 phage library must have diverse cDNA sequences for expression of the peptides to be displayed at a multiple cloning site within the gene encoding the capsid protein. For this reason, two T7 phage libraries were prepared from the cDNA of human leukocytes and *Drosophila melanogaster*. The biotinylated derivative of CLA (**6**) was immobilized on a streptavidin-coated well and then incubated with either of the two T7 phage libraries. Phage selected after each round of the biopanning procedure was extensively washed with buffer
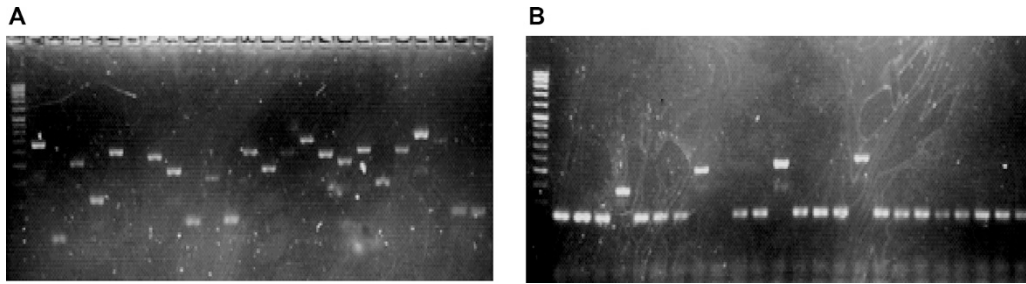
Fig. 2 Agarose gel electrophoretic analyses of the inserted Drosophilae cDNAs in T7 phage selected by biopanning.

A: PCR amplified cDNAs after three rounds, B: PCR amplified cDNAs after four rounds. After four rounds of selection, 89% of the selected phage exhibited similar-sized bands by agarose gel electrophoresis.

Table 1 The selected peptide and human p8

```
19aa    --------------------------------------------------------NSPA
P8      MATFPPATSAPQQPPGPEDEDSSLDESDLYSLAHSYLGGGGRKGRTKREAAANTNRPSPG
                                                                   **.


19aa    GISRELVDKLAAALE-------
P8      GHERKLVTKLQNSERKKRGARR
        *  .*:** **   :  .
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The selected 19aa had a sequence similarity with those of human p8 protein residues 58~70 of 82 searched by FASTA program against Swiss Prot protein data base.

to eliminate adventitiously bound clones. The eluted phage were recovered and tested for affinity to biotinylated-CLA (**6**). Four rounds of biopanning were carried out, and the phage titer determined for the washed and eluted solutions after each round. After the fourth round of panning the ligand-selected and recovered phage represented 0.78% of the leukocyte library and 0.23% of the Drosophilae library, whereas the recovered phage from the control was negligible in both cases.

Sequence Analysis of Ligand-selected Phage and Homology Search of Peptide Sequences

The cDNA inserts from recovered phage were amplified by the polymerase chain reaction (PCR) for analysis by agarose gel electrophoresis and DNA sequencing. After the fourth round of selection (Fig. 2B), the recovered cDNA inserts were much more uniform than the products from the third round (Fig. 2A). In the case of the *D. melanogaster* phage library, 89% of the 83 sequenced clones after the fourth round were identified as the same sequence of 57 nucleotides. Furthermore, this was also obtained as a major sequence from the human leukocyte library (*i.e.* 50~60% of sequenced clones). The selected nucleotide sequence and its translation product (NSPAGISRELVDKLAAALE) were screened against the Swiss Prot protein database using the

FASTA program [10]. The search revealed a 66% similarity (from 13 residues of the query sequence) to a portion of the human p8 protein (residues 58~70 of 82) (Table 1). The p8 protein was found overexpressed in many human cancers [11]. An immunohistochemically investigation of p8 expression in 50 cases of breast carcinoma indicated that high expression of p8 was observed in 60% of cases [12]. In the case of medullay thyroid, 43% of cases were regarded as high group p8 expression. The p8 expression level was directly linked to lymph node metastasis [13]. In addition to those, p8 is a new target of gemcitabine, the only available chemotherapeutic treatment of pancreatic cancer, in pancreatic cancer cells [14]. There is only presumptive evidence for the relationship of p8 and CLA yet; however, p8 could be a candidate for CLA binding target of anti-cancer property from those evidences. Actually, phase II trial of CLA and pamidronate therapy in myeloma has been evaluated [15].

Surface Plasmon Resonance Analysis

Binding analyses between macrolides and a synthetic peptide (NSPAGISRELVDKLAAALE) were performed by surface plasmon resonance. The synthetic peptide was fixed on a CM5 sensor chip to capture the peptide on the carboxymethyl dextran matrix. The dissociation constants
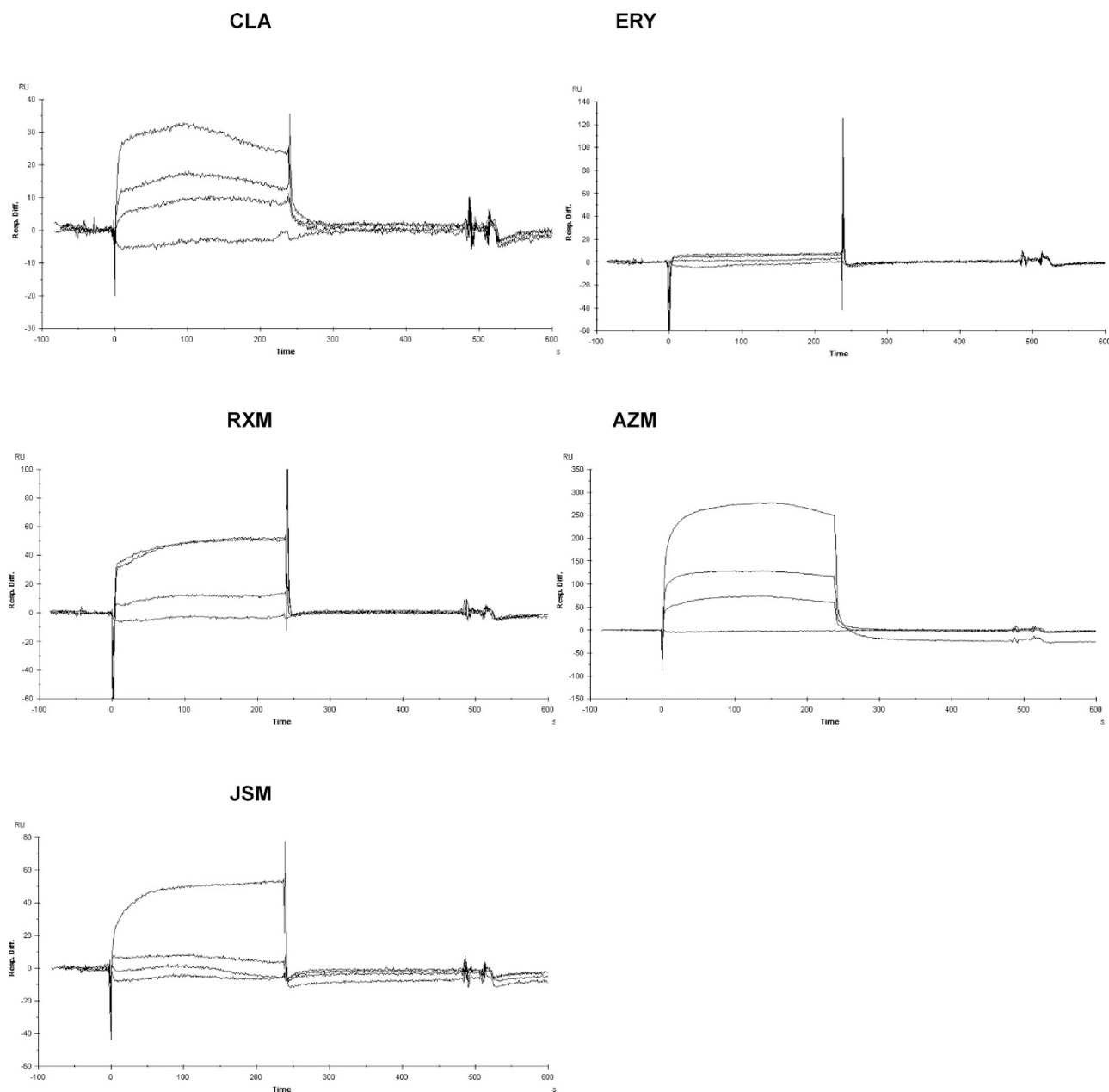
Fig. 3 SPR (BIAcore) analyses of CLA, ERY, RXM, AZM and JSM to the synthetic peptide from T7 phage display libraries.

Bindings of the macrolide antibiotics to the synthetic peptide were detected by surface plasmon resonance (SPR) signals (BIAcore, see Experimental Section), and were indicated in response units. Three different concentrations of each macrolide (see text) were injected over the immobilized peptide (4700 RU) on a CM5 sensor chip for 3 minutes at 20 ml/minute and dissociated for 120 seconds at 20 ml/minute.

(K_D) of the macrolides with the peptide were determined to be 2.1×10^{-3} M with CLA, 5.4×10^{-3} M with ERY, 6.2×10^{-5} M with RXM, 1.1 M with AZM and 3.4×10^{-2} M with JSM, respectively, using the BIA evaluation 3.1 software (Biacore AB).

Discussion

The selected nucleotide sequence revealed a similarity to the residues 58~70 of 82 amino acids in the human p8 protein (Table 1). The p8 protein is a small-stress protein involved on several cellular functions including apoptosis. Prothymosin alpha (ProTalpha) and p8 are two natively unstructured proteins with anti-apoptotic activity. Their

interaction results in the formation of heterodimer complex with stable structure. When Hela cell were treated with staurosporine, the effects of knocking down p8 and/or ProTalpha or over-expressing p8 and/or ProTalpha on caspases 3/7 and 9 activities and on cell death were monitored. Transfecting ProTalpha or p8 small interfering RNAs increased the activities of both caspase and the number of apoptotic nuclei. However, transfecting both small interfering RNAs resulted in no further increase. Over-expressing p8 or ProTalpha did not alter caspase activities, whereas over-expressing both resulted in a significant reduction of caspase activities [16]. Over-expression of was obtained by transfection of apoptotic vectors and inhibition by using specific siRNAs [17]. Inhibition of apoptosis correlated with the level of ProTalpha with lowest concentration, demonstrating that the anti-apoptotic was borne by the complex of p8/ProTalpha, the two proteins, being individually inactive. The stress-regulated protein p8 (also deigned as candidate of metastasis 1) was also identified as an essential mediator of cannabinoid antitumoral action and show p8 upregulation is dependent on de novo-synthesized ceramide [18]. It is also observed that p8 mediates its apoptotic effect *via* upregulation endoplasmic reticulum stress-related genes ATF-4, SHOP, and TRB3.

We have used a phage display technique to screen for human proteins that specifically bind to CLA. Affinity guided biopanning using biotinylated CLA selected a peptide composed of 19 amino acids. SPR experiments showed that the rank order of affinity is RXM>CLA>ERY>JSM>AZM. The FASTA search revealed a partial similarity to the human p8 protein (Table 1). Human p8 protein was over-expressed in many human cancers [11], including breast carcinoma [12] and medullay thyroid [13], and also contributes to the metastatic phenotype [18], although its function has not yet been fully elucidated. Thus, if CLA and other macrolides antibiotics could bind to p8 protein and then interrupt the formation of p8/ProTalpha, it might regulate their apoptotic activity *in vivo*. Phase II trial of CLA and pamidronate therapy in myeloma [15] could imply the existence of other human proteins that specifically bind to macrolide antibiotics. The binding analyses between human p8 and macrolide antibiotics confirm binding of clarithromycin to p8 and this will be published elsewhere.

The results obtained here indicated that T7 phage display method might be useful to determine relatively weak interactions between small molecule drugs and the selected peptides which could also display a possible binding site conserved in binding proteins.

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