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Synthesis of a biotinylated camptothecin derivative and determination of the binding sequence by T7 phage display technology

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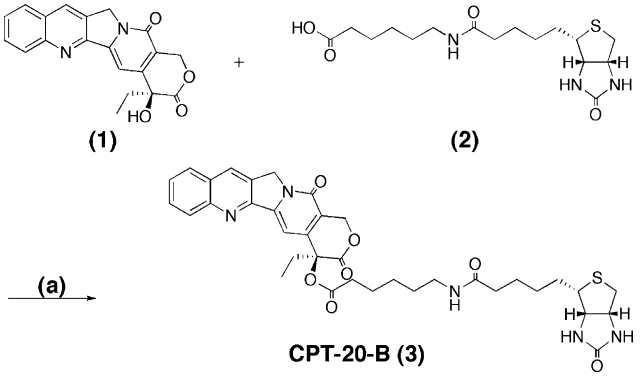
Abstract—A biotinylated derivative of the anti-tumor agent camptothecin (CPT) was synthesized and used in a phage display assay to identify drug-binding sequences. After three rounds of selection using C20-biotinylated CPT (CPT-20-B) as bait, a CPT-20-B-binding sequence, NSSQSARR, was identified.

1. Introduction

Camptothecin (CPT) is an anti-tumor compound isolated from *Camptotheca acuminata* in 1966 (Scheme 1).[1] Irinotecan (Iri), a CPT derivative with clinical applications, is a water-soluble prodrug, which is metabolized to SN-38 by carboxylesterase.[2,3] Topoisomerase I (top I) was originally reported to be the molecular target of CPT,[3,4] which showed reversible stabilization of top I–DNA cleavage complexes with CPT or SN-38.[5,6] However, reports of several other biological activities of this drug suggest that there are one or more unknown targets, such as membrane or cytoplasmic proteins, in addition to top I.[7–12] Epidemiological studies have indicated that NSAIDs, especially COX-2 inhibitors, enhance the anti-tumor activities of Iri and other anti-tumor drugs in combination therapy.[13–15] The identification of CPT binding targets could elucidate additional sites of action that are responsible for the anti-tumor activity and/or associated side effects of CPT.

To screen for potential binding targets, we focused on phage display technology. This is a powerful tool for the rapid determination of molecular targets, providing direct information about binding domains.[16] A random



Scheme 1. Synthesis of CPT-20-B. Reagents and condition: (a) EDC, DMAP in DMF at 50 °C.

cDNA is inserted into the multi-cloning region of a phage vector to construct a phage library, which displays cDNA-derived fusion peptides on the surface of the capsid. Using a phage library and biotinylated molecules immobilized on a streptavidin-coated carrier as bait, phage particles may be screened. Recovered phage can then be amplified by infection of a log-phase culture of *Escherichia coli*. Following several rounds of selection, the remaining phage particles are disrupted to extract the phage DNA. After amplifying the inserted cDNA by the polymerase chain reaction (PCR), the fragments

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are sequenced to determine the corresponding amino acid sequence displayed on the phage capsid. Peptides that specifically bind to the bait can then be used in a homology search to identify the likely molecular target. Using this technology, we recently determined the NK109-binding peptide and protein.[17]

Here, we report the synthesis of a biotinylated derivative of CPT and identification of a binding peptide by T7 phage display technology.

2. Results and discussion

2.1. Synthesis of biotinylated CPT derivative

CPT-20-B (**3**) was prepared from a condensation reaction of commercial CPT (**1**) and 6-biotinylaminocaproic acid (**2**) with EDC/DMAP in DMF at 50 °C (Scheme 1).

2.2. Affinity selection with phage display method

The binding peptide sequences of CPT were validated by phage display using a biotinylated derivative, CPT-20-B (**3**) (Scheme 1), and a T7 phage library constructed from human leukocyte cDNA by a random primer strategy (Novagen, Madison, WI, USA).[18,19] An aliquot (10^9 pfu) of the amplified phage library was allowed to bind to 1 nmol of the biotinylated CPT derivative (**3**) immobilized on a streptavidin-coated 96-well plate. After washing 10 times with 200 μl of 100 mM Tris–HCl (pH 7.5) to remove non-specifically bound phage, the remaining phage particles were either eluted five times with 200 μl of elution buffer (3% Tween 20 in 100 mM Tris–HCl, pH 7.5) or used to infect a log-phase culture (200 μl) of *E. coli*. On this method, an elute condition that could dissociate the binding phage from the immobilized bait is often mattered to determine, especially screen for small molecules. Therefore, direct infection using host cell solution was used to recover the remaining phage on second and third round. After three rounds of selection using CPT-20-B, the cDNA inserts from an arbitrarily selected 40 phage particles were respectively amplified by PCR and analyzed by 1% agarose gel electrophoresis (Fig. 1). The cDNA sequences were obtained, and 10 of the cDNAs that showed the same mobility on agarose (Fig. 1, •) were found to encode part of a Ca^{2+}-binding protein. However, the random primer strategy appeared to have generated a frame-shifted stop codon, thereby giving a shorter peptide than would normally be expected. The peptide obtained from these 10 clones, NSSQSARR, was encoded in-frame and displayed fusion peptides part of the capsid.

2.3. Forward affinity check

The binding of the selected clones (NSSQSARR) was compared with that of control phage particles (NSPAGISREVDKLAAALE) by a forward affinity check (Fig. 2A). Each phage particle was incubated with CPT-20-B immobilized on a streptavidin-coated 96-well microplate. After washing to remove non-specifically bound clones, binding phages were eluted. As shown in Figure 3A, the recovery rate for the selected phage particles was 2.5-fold higher than that of control clones. The remaining 30 phage particles did not show specific binding to CPT-20-B on forward affinity check, demonstrating that these clones represented a false-positive background.

2.4. Reverse affinity check

A reverse affinity check was also carried out to confirm the specificity of the binding (Fig. 2B).[20] Phage clones were immobilized on a nitrocellulose membrane and incubated with CPT-20-B, followed by alkaline phosphatase (AP)–avidin binding and reaction with an AP substrate. Intensity rates of selected clones were approximately 3.3-fold stronger than those of control phage particles, agreeing with the result of the forward affinity check (Fig. 3B). A comparison of intensity rates between selected and control phage without CPT-20-B showed no difference, confirming that the selected clone bound to CPT-20-B specifically (Fig. 3C). An analysis of non-biotinylated CPT binding to the synthetic NSSQSARR will be reported elsewhere.

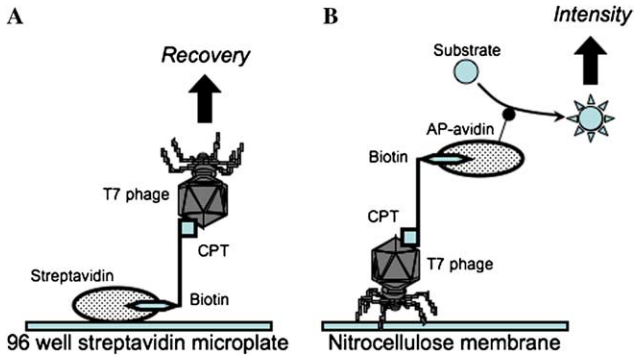

Figure 2. Forward and reverse affinity checks. (A) Forward affinity check: an aliquot of phage clone was allowed to bind to biotinylated CPT derivative immobilized on a streptavidin-coated 96-well microplate. The binding phage was eluted with various elution buffer or recovered by infection with *E. coli*. (B) Reverse affinity check: phage clone immobilized on nitrocellulose membrane was incubated with or without biotinylated CPT derivative and followed by AP–avidin binding.

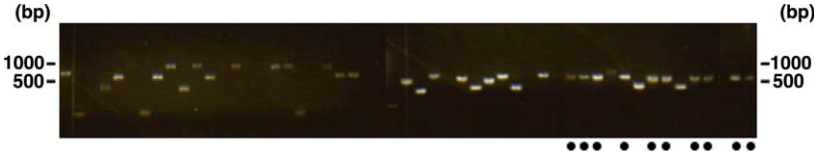

Figure 1. Agarose gel (1%) electrophoresis. After three rounds of selection, the cDNA inserts from a set of arbitrarily selected 40 phage particles were amplified by PCR and analyzed by electrophoresis. (•) These cDNAs encoded the peptide NSSQSARR as a fusion peptide of the capsid protein.

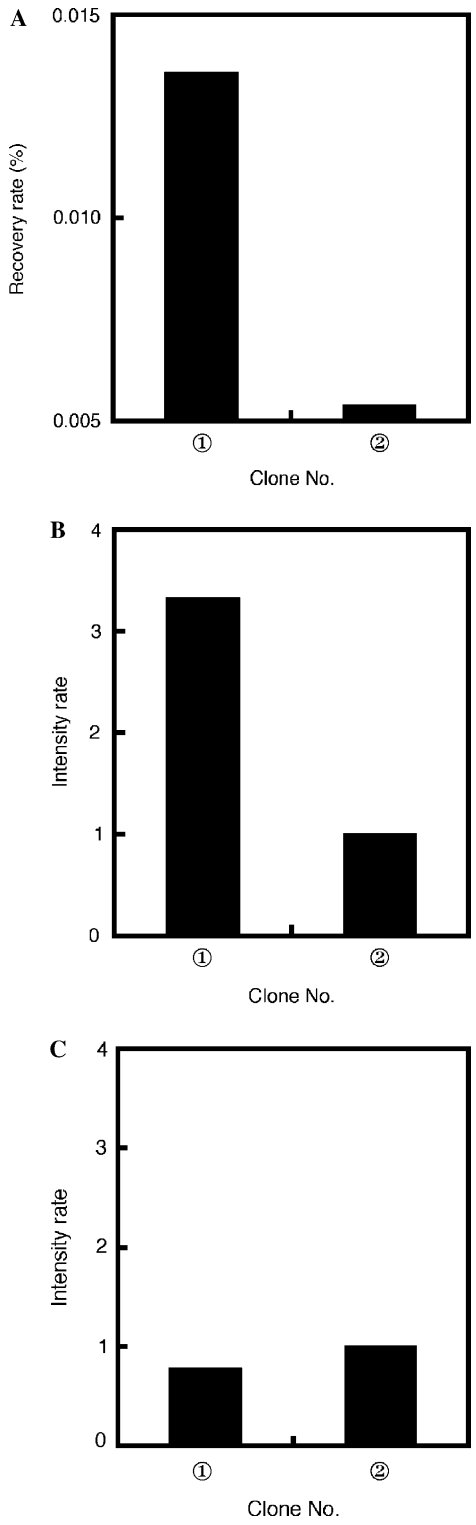

Figure 3. The results of forward and reverse affinity checks against CPT-20-B. (①) Phage clone expressing NSSQSARR. (②) Control clone expressing NSPAGISREVDKLAAALE. (A) Forward affinity check: approximately 10^9 pfu of the mono clone was mixed with immobilized CPT-20-B (1 nmol). After washing, the eluted phage titer was determined and displayed as the recovery rate. Recovery rate (%) = [titer of the eluted fraction (pfu)/titer of the input (pfu)] × 100. (B) and (C) Reverse affinity check. Phage clone immobilized on a nitrocellulose membrane was incubated with (B) or without (C) 100 μM of CPT-20-B, AP–avidin at 1000-fold dilution, respectively. BCIP/NBT was used as the AP substrate for detection. Intensity rate was calculated from the NIH image. pfu: Plaque forming unit.

3. Materials and methods

3.1. Synthesis of biotinylated CPT derivative

Commercial CPT (**1**) (19.3 mg, 0.055 mmol) was reacted with 6-biotinylaminocaproic acid (**2**) (18.8 mg, 0.053 mmol) with EDC (35.2 mg, 0.188 mmol)/DMAP (8.9 mg, 0.073 mmol) in dried DMF (3 ml) at 50 °C (Scheme 1). The reaction mixture was stirred for 4 h at rt. The resulting mixture was charged on a silica gel column ($\phi 10 \times 60$ mm, $CHCl_3$/MeOH = 9/1) and purified to give CPT-20-B (**3**) (5.1 mg, 0.007 mmol, 13.5%) as a pale yellow powder. The reaction product was analyzed by NMR and ESIMS. NMR data were obtained from JEOL JNM-LA400 (400 MHz for ^1H, JEOL, Tokyo, Japan) in a mixture of CD_3OD and $CDCl_3$. Chemical shifts were expressed by δ parts per million using TMS as an internal standard. Mass spectral data was collected on an ABI QSTAR (Applied Biosystems Japan (ABI), Tokyo, Japan) with ESI in the positive ion mode.

CPT-20-B: $C_{36}H_{41}O_7N_5S$; pale yellow powder; R_f = 0.43 ($CHCl_3$/MeOH = 9/1); ^1H NMR (1:1 CD_3OD/$CDCl_3$); δ 1.02 (3H, t, J = 7.3 Hz), 1.36–1.39 (4H, m), 1.49–1.70 (8H, m), 2.12 (2H, t, J = 7.3 Hz), 2.20 (2H, m), 2.57 (2H, t, J = 7.3 Hz), 2.67 (1H, d, J = 12.4 Hz), 2.88 (1H, dd, J = 5.1, 12.9 Hz), 3.0–3.20 (2H, m), 3.60 (1H, m), 4.26 (1H, dd, J = 4.4, 7.8 Hz), 4.45 (1H, dd, J = 4.4, 7.8 Hz), 5.30 (2H, s), 5.46 (1H, d, J = 16.8 Hz), 5.60 (1H, d, J = 16.8 Hz), 7.35 (1H, s), 7.69 (1H, t, J = 7.1 Hz), 7.85 (1H, t, J = 7.1 Hz), 8.04 (1H, d, J = 8.3 Hz), 8.15 (1H, d, J = 8.6 Hz), 8.60 (1H, s); HRMS calcd for $C_{36}H_{42}O_7N_5S$, $(M+H)^+$ m/z: 688.2805; found, 688.2802.

3.2. Construction of a T7 phage library from human leukocytes

The T7 phage library was constructed according to the manufacturer's instructions (Novagen).[17,18] Briefly, poly(A) + RNA, random primers, 5′-methylated dCTP, T4 DNA polymerase, *Eco*RI/ *Hind*III linkers, *Eco*RI, *Hind*III, a T7 select vector and T7 Packaging Extracts were used. Aliquots (80 μg) of total RNA, extracted from human leukocytes, were used to construct the cDNA library. Oligotex-dt30 <super> (Takara, Shiga, 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 using random primers. 5′-Methylated dCTP was then incorporated into both strands, without extraction or precipitation between the first and second strand syntheses. The cDNA was then treated with T4 DNA polymerase to generate flush ends and ligated with directional *Eco*RI/*Hind*III linkers. Following linker ligation, the cDNA was digested sequentially with *Eco*RI and *Hind*III and then was inserted into T7Select10-3b vector arms at the corresponding restriction sites. The cDNA was cloned into the *Eco*RI/*Hind*III sites of the T7 phage 10-3b vector and packaged into the phage. The titer of this library was 9.4×10^{10} pfu/ml.

3.3. Phage affinity selection and DNA sequence analysis

An aliquot (10^9 pfu) of the amplified phage was allowed to bind to 1 nmol of biotinylated CPT derivative immobilized on a streptavidin-coated 96-well microplate by rotating gently for 1 h. The wells were then blocked with 3% skimmed milk in 100 mM Tris–HCl (pH 7.5). After washing 10 times with 200 μl of 100 mM Tris–HCl (pH 7.5) to remove non-specifically bound phage, the remaining phage particles were either eluted five times with 200 μl of elution buffer (3% Tween 20 in 100 mM Tris–HCl, pH 7.5) or used to infect a log-phase culture (200 μl) of *E. coli*. Following three rounds of selection, 40 plaques were randomly picked from LB plates and each was dissolved in phage extraction buffer (100 mM NaCl, 6 mM $MgSO_4$ in 20 mM Tris–HCl, pH 8.0). The candidate clones were amplified and their affinity to biotinylated CPT derivative was checked. The phages were disrupted by heating the extract to 65 °C for 10 min. After amplifying the phage DNA by PCR, the fragments were purified with ExoSAP-IT and EtOH precipitated. The fragments were sequenced on an ABI Prism3100 Genetic Analyzer (ABI) to determine the corresponding amino acid sequence displayed on the T7 phage capsid.

3.4. Forward affinity check

A forward affinity check was performed using phage affinity selection as described above.

3.5. Reverse affinity check

The enriched population of plated candidate phage particles was transferred onto a nitrocellulose membrane. After washing with TBST, the membranes were blocked with TBST containing 3% skimmed milk, rotating gently for 1 h at room temperature. The membranes were then incubated with 100 μM of biotinylated CPT derivative (1% DMSO in TBST) at 4 °C overnight and then washed three times with 1% DMSO in TBST (each for 5 min). AP-conjugated avidin (Sigma, St. Louis, MO) was added at 1000-fold dilution and incubated for 1 h at room temperature. The membranes were washed as described previously. Signals were detected by adding BCIP/NBT solution (Wako, Osaka, Japan). The density of the spots were determined by using NIH image (http://rsb.info.nih.gov/nih-image).

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