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Camptothecin binds to a synthetic peptide identified by a T7 phage display screen

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Abstract—An analysis of non-biotinylated camptothecin (CPT) binding to the C-20-biotinylated CPT binding peptide NSSQSARR was carried out using two methods, quartz-crystal microbalance (QCM) and surface plasmon resonance (SPR). The peptide was immobilized peptide on a sensor chip and showed a dissociation constant (K_D) of approximately 0.1 μM against CPT in QCM and SPR experiments.

1. Introduction

Recently, rational molecular design based on docking simulations has emerged as a major approach for generating effective anti-tumor agents. The drugs Gefinitib (ZD1839, Iressa) and Imatinib (STI571, Gleevec), which are used for chemotherapy to treat non-small cell lung cancer and chronic myeloid leukemia respectively, are typical examples developed by this approach.[1–3] Target validation is imperative at the outset of the drug development process to minimize possible side effects. In addition, physiological information, such as the dissociation constant (K_D) value, or binding information deduced from simulations of docking between compounds and their cognate binding domains is useful for molecular drug design.

As discussed in a preceding article, we identified a CPT-20-B-binding peptide sequence, NSSQSARR, by T7 phage display screen.[4] This sensitive method enables the rapid determination of molecular targets from a diverse range of proteins, including those that are membrane-associated or subject to rapid turnover. Furthermore, sets of small molecules, such as biotinylated derivatives, can be used as baits, providing direct information about the binding domains in larger proteins. To date, only a few examples have been reported of target validation using functional small molecules in phage display technology.[5–11]

Here, we carried out a kinetic analysis of QCM and SPR experiments measuring the binding of non-biotinylated CPT to a synthetic CPT-20-B-binding peptide. We also describe a docking simulation carried out using Insight II/Discover program (Accelrys Inc., San Diego, CA, USA).

2. Results and discussion

2.1. Kinetic analysis by QCM

A 27-MHz QCM (AffinixQ, Initium Inc., Tokyo, Japan) was employed to analyze the interaction between CPT and peptide.[12,13] The synthetic peptide was immobilized on a ceramic sensor chip using an amine coupling reaction. Four different concentrations of CPT were added to the peptide immobilized on the ceramic sensor chip with a gold surface. The binding of CPT to this peptide was calculated by monitoring the alterations in frequency (ΔF) resulting from changes in mass on the electrode surface. As shown in Figure 1A, the frequency decreased after injecting each concentration of CPT, confirming that CPT binds to this peptide. Linear-reciprocal plots showed linearity, indicating that CPT showed Langmuir

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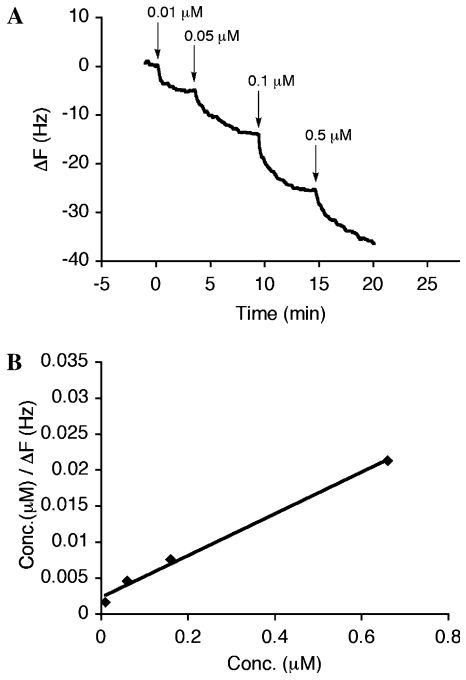

Figure 1. QCM analysis (AffinixQ). Four different concentrations of CPT (0.01, 0.05, 0.1, and 0.5 μM) were added to a cuvette in which they interacted with peptide immobilized on a gold electrode surface of a ceramic sensor chip. (A) Interaction of CPT with NSSQSARR. 1 Hz = 30 pg. (B) Linear-reciprocal plot of concentration (μM)/Δ*F* (Hz) against various concentrations of CPT.

type 1:1 binding to the peptide (Fig. 1B). The K_D value of CPT binding to the peptide, NSSQSARR, was 94 nM.

2.2. Kinetic analysis by SPR

An SPR biosensor, an interaction analysis instrument of the flow injection type, was also employed to analyze the interaction between CPT and peptide. The resulting K_D value was compared to that obtained by QCM, which employs a cuvette-type analytical apparatus. Five different concentrations of CPT were used to measure the binding of CPT to the peptide, which was immobilized on a CM5 sensor chip by amine coupling. CPT bound to the peptide, showing low rates of association and dissociation. The kinetic constants for the interaction were determined by fitting the SPR association and dissociation curves obtained at various concentrations of CPT. K_D values were calculated by global fitting using BIAevaluation 3.2 software. The resulting K_D value was 112 nM (Table 1). Result from the SPR analysis agrees to that obtained by QCM, confirming the binding between CPT and NSSQSARR.

Table 1. K_D values for binding of CPT to the peptide NSSQSARR obtained from QCM and SPR analyses

	QCM[a]	SPR[b]
K_D (nM)	94	112

[a] Value was calculated from linear-reciprocal plot by using AQUA ver1.5 software (Initium Inc.).
[b] Value was calculated from global fitting by using BIAevaluation 3.2 software (BIAcore).

2.3. Docking simulation of CPT binding to the NSSQSARR peptide

A docking simulation using InsightII/Discover (Accelrys Inc.) was carried out to further investigate the interaction between CPT and NSSQSARR. Results indicated that the polycyclic moiety of CPT fits into a complementary groove formed by the peptide and that hydrogen bonding strengthens these interactions, in particular hydrogen bonds that form (1) between the carbonyl oxygen of Arg8 and the hydroxyl group at C-20 of CPT, and (2) between the guanidine moiety of Arg7 and the carbonyl oxygen at C-16a of CPT (Fig. 2).

We are currently validating the candidate protein of CPT from a homology search using NSSQSARR as a query followed by binding analysis with QCM and SPR.

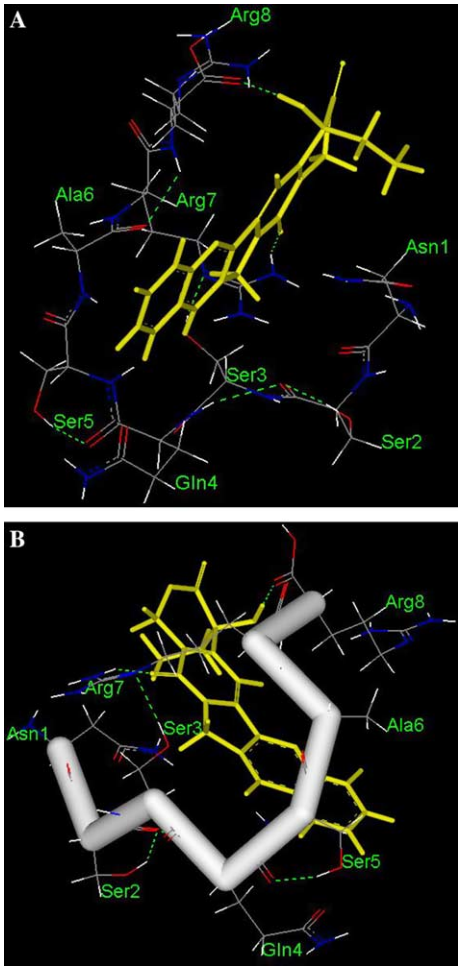

Figure 2. (A, B) Docking simulation of CPT with NSSQSARR, the peptide identified using a phage display method. The structure of CPT is shown in yellow. The atoms comprising the structure of NSSQSARR are color coded: carbon in gray; hydrogen in white; oxygen in red; nitrogen in blue. The green dashed lines indicate hydrogen bonding. This figure was prepared using Insight II/Discover (Accelrys Inc.). (B) Peptide backbone is shown in white.

3. Materials and methods

3.1. Peptide synthesis

The proposed CPT-20-B-binding peptide (NSSQSARR), which was identified by a phage display screen,[4] was synthesized by the Fmoc method using a peptide synthesizer PS-3 (Aloka, Tokyo, Japan). Fmoc-Arg (4.3 g, 6.5 mmol; Carbiochem, San Diego, CA, USA) was reacted with DCC (0.64 g, 3.1 mmol) in CH_2Cl_2 (25 ml) for 10 min at room temperature to synthesize Fmoc-Arg anhydride [$(Fmoc\text{-}Arg)_2O$]. After filtration through celite and concentration in vacuo, $(Fmoc\text{-}Arg)_2O$ was reacted with Wang-resin (75–150 μm, 1.6 g, 1.65 mmol; Wako, Osaka, Japan) with DMAP (95 mg, 0.778 mmol) in dried DMF (10 ml) for 1 h at room temperature on a shaker to produce an Fmoc-Arg–Wang-resin. By using 200 mg (0.2 mmol) of this resin and adding 0.1 mmol of each amino acid (Carbiochem) sequentially, the peptide chain (NSSQSARR) was extended from the C terminal to the N terminal end by repeating the process of Fmoc deprotection using 20% piperidine in DMF (6 ml), activation by HBTU (75.8 mg, 0.1 mmol) and 0.4 M 4-methylmorpholine in DMF (3 ml), and amino acid coupling with the resin. The resultant material was treated with 5 ml of cleavage cocktail (0.75 g phenol, 0.25 ml 1,2-ethanedithiol, 0.5 ml thioanisole in 10 ml of 95% TFA) to cleave the peptide from the resin and deprotect the side chains. After cold ether precipitation, the precipitant was washed with ether 3 times and recovered for HPLC purification.

3.2. Peptide purification

The peptide was purified using a reverse phase preparative HPLC instrument (SSC-3461, Senshu Scientific, Tokyo, Japan) equipped with a CAPCELL PAK C-18 column ($\phi 20 \times 250$ mm, UG 120 Å, Shiseido, Tokyo, Japan) that was kept at 40 °C. A binary gradient with a flow rate of 4 ml/min was employed; A phase: 0.1% TFA aq, B phase: 0.1% TFA in acetonitrile. The gradient condition was 5% B (0 min) to 20% B (20 min), and it was then held at 20% B for 10 min. The UV absorption at 210 nm was monitored using a UV detector (SSC-5200, Senshu Scientific). The peak detected at 20 min was fractionated and dried up to obtain the purifed NSSQSARR. The identity of the peptide was verified by TOFMS [ABI QSTAR, Applied Biosystems Japan (ABI), Tokyo, Japan].

3.3. Kinetic analysis by QCM

Binding analysis of the interaction between CPT and synthetic peptide was performed with a 27 MHz QCM (AffinixQ).[10,11] The synthetic peptide was immobilized on a ceramic sensor chip by an amine coupling reaction. A drop of 5 mM DTDP in EtOH was applied to a sensor chip to immobilize the DTDP directly on the gold electrode surface of the chip by a Au–thiol interaction. The sensor chip was then activated by adding a coupling solution including 26 mM EDC and 43 mM NHS. After immersing the sensor chip in 8 ml of saline solution (150 mM NaCl in 50 mM phosphate buffer, pH 7.0), the synthetic peptide was added to the buffer and the immobilization was followed by monitoring alterations in frequency (ΔF) resulting from changes in mass at the electrode surface. This immobilized peptide generated a signal of about 80 Hz, indicating that about 2.4 ng of synthetic peptide was bound. CPT (0.01, 0.05, 0.1, and 0.5 μM) was then added in buffer (150 mM NaCl in 50 mM phosphate buffer, pH 7.0, 10% DMSO) at 25 °C. AQUA ver1.5 software (Initium Inc.) was then used to determine the kinetic parameters.

3.4. Kinetic analysis by SPR

Binding analysis between CPT and synthetic peptide was also performed with a SPR biosensor (BIACORE 3000, BIAcore, Uppsala, Sweden). The synthetic peptide (200 μg/ml, 170 μl) in 10 mM carbonate buffer, (pH 8.5) was injected over a CM5 sensor chip at 10 μl/min captured on the carboxymethyl dextran matrix with an amine coupling reaction. The surface was activated by injecting a solution containing 200 mM EDC and 50 mM NHS for 14 min. The peptide was injected and the surface was then blocked by injecting 1 M ethanolamine at pH 8.5 for 14 min. This reaction immobilized about 500 resonance units (RU) of synthetic peptide. Binding analysis of CPT was performed in buffer (150 mM NaCl in 50 mM phosphate buffer, pH 7.0, 8% DMSO) using a flow rate of 20 μl/min at 25 °C. BIAevaluation 3.2 software (BIAcore) was used to determine the kinetic parameters.

3.5. Docking simulation to show formation of a complex between CPT and NSSQSARR

An initial three-dimensional structure of CPT was generated and then refined by energy minimization using the molecular modeling software Insight II/Builder (Accelrys Inc.). All calculations were conducted on an HP workstation wx4100 (3.4 GHz processor and 1024 MB of memory), running under the Red Hat Enterprise Linux WS2.1 operating system. The initial conformation of NSSQSARR was refined by energy minimization using Insight II/Biopolymer (Accelrys Inc.) and the molecular docking simulation of CPT binding to NSSQSARR was performed in Insight II/Discover using the consistent-valence forcefield (CVFF). All torsions except the peptide bonds were unconstrained during the docking procedure. After docking had been carried out, the lowest energy docked structure was analyzed.

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