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Determining for the Interaction of Constitutive Androstane Receptor and CITCO Using a Surface Plasmon Resonance Based Biosensor System

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Abstract: This study investigated the binding affinity of constitutive androstane receptor (CAR) with its activator, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime (CITCO) in order to develop a rapid method for screening of protein-binding compounds. At first, the performance capacity of surface plasmon resonance (SPR) system was confirmed by the interaction of commercial carbonic anhydrate II (CAII) protein and 4-carboxybenzenesulfonamide (CBS) compound. The target protein, ligand binding domain of human CAR (hCAR), was optimally immobilized on SPR sensor chip using amine coupling method and then used to detect the interaction with chemical molecules. CITCO, known as a hCAR agonist in previous studies was used as the positive control to develop the method for determination of the binding affinities between SPR-immobilized CAR proteins and chemicals. As expected, CITCO showed specific bindings to hCAR protein in this study, indicating the application potential of SPR system in screening probable ligands of proteins.

Keywords: Constitutive androstane receptor, CITCO, surface plasmon resonance.

1. Introduction

The constitutive androstane receptor, also known as nuclear receptor subfamily 1, group I, member 3 is a protein encoding by the NR1I3 gene (CAR, NR1I3) [1]. CAR functions as the sensor of endogenous and exogenous compounds, regulating the expression of functional proteins which account for the metabolism, transportation and excretion of these substances from the body [2-4]. Hence, CAR is important in the detoxification of foreign substances such as drugs and environmental pollutants [5, 6]. Moreover, pathological researches showed that CAR relates to tumor development and cancer [7], diabetes and obesity [8] diseases by ligand-induction. Although the mechanism of action of exogenous substances to organisms through the CAR has been extensively studied, but most

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studies are performed in *in vitro* experiments with reporter genes (based on CYP gene expression). However, this method could not show the initial attack mechanism of molecules to organisms, meaning that it has not displayed the specific role of the CAR in response to exogenous substances [6]. Research on the interaction of the potential ligands with the receptor will contribute to completing the picture of the attack mechanism of foreign molecules to organisms from the first step and clarifying whether a molecule can interact directly or indirectly to the receptor. So far, the method to determine the binding ability of the ligand with the CAR is limited. Experiment on the binding ability of CAR with ligands was first performed by Moore et al. (2000) based on the principle of fluorescence resonance energy tranfer between the chromophore-marked molecules. This experiment requires interactive molecules that are labeled with biotine and takes time for incubation with the target receptor. With the goal to rapidly screen for effective potential compounds of organisms through CAR, development of a method to rapidly detect ligands of CAR is necessary. Surface plasmon resonance (SPR) biosensor, a novel analytical instrument that is a multiplex optical biosensor was used to monitor bimolecular interactions without labelling the molecules in real time through a SPR-based detector. This technology is able to measure directly and rapidly the interaction of small molecules with immobilized macromolecular targets [9, 10]. This study selected the SPR system as an object to develop a biosensor system for a rapid screening for potential ligands of CAR.

2. Materials and Methods

Testing of instrument: CAII protein and CBS compound (Bio-Rad) were used to test the performance capability of SPR system. The immobilization of CAII protein using amine coupling method and the interaction of CAII with CBS were conducted on SPR sensor chip (Reichert) as described in the previous reports [10, 11] with the conditions described in Table 1.

Table 1. Summary of interaction conditions and binding affinities of CAII and CBS

Protein Immobilization (RU)	**CBS (MW= 201) concentration (µM)**	**Kinetic**		**Equilibrium**		**References**
		k_a **(1/ms)**	k_d **(1/s)**	K_D **(µM)**	K_D **(µM)**	
CAII (21,400 ± 500)	0, 0.08, 0.25, 0.75, 2.22, 6.67, 20	1.74×10^4	0.04	2.2 ± 0.3	3.2 ± 1.3	This study
	0, 0.08, 0.25, 0.75, 2.22, 6.67, 20	2.83×10^4	0.03	1.2	ND	Turner (2008)
	0, 0.08, 0.25, 0.75, 2.22, 6.67, 20	1.90×10^4	0.03	1.6	ND	Bravman (2006)

His-hCAR and CITCO interaction: Recombinant His-tag ligand binding domain (LBD) of hCAR (Jena Bioscience) was immobilized by amine coupling method in running Hepes buffer (0.01M HEPES, 0.15M NaCl, 0.003M EDTA, 0.005% Tween 20, pH 7.4) [9]. CAR was prepared at 50µg/ml concentration in Na acetate buffer (10mM, pH 5.0) and was injected for 10 min at 25µl/min over the activated channel. The interaction of CITCO and hCAR was optimized through testing under different conditions of concentration, injection flow rate and contact time (Table 2). The PBS-T buffer (0.02M Na_2HPO_4, 0.15M NaCl, 0.001M dithiothreitol, 0.005% Tween 20, pH 7.4 and 5% DMSO) was used as running buffer and chemical dilution buffer [9]. Triplicate injections of each concentration were done to check the reproducibility.

Data Analysis: Binding curves were processed by aligning the baseline with start injection signals, and by subtracting signals of an activated and blocked reference channel. The binding affinity was evaluated by equilibrium dissociation constant (K_D) drawn from the responses of the six analyte concentrations. Responses were fitted to a simple bimolecular equilibrium model at 50% saturation response. K_D is given for a specific ligand binding to CAR. No K_D was given for non-specific binding of the chemical with a maximum plateau not achieved from dose-dependent responses. K_D value is high then the binding affinity is low. Obtained data were analysed using GraphPrism software.

3. Results and Disscussion

3.1. The interaction of CAII protein and CBS compounds on SPR system

CAII that is known as a standard protein was used for amine coupling immobilization in this study [11]. The immobilization level of CAII reached at 21,400 ± 500 RU (Fig. 1). CBS, a small molecule that was reported as a ligand of CAII [11] was injected over the CAII channel with different concentrations (Table 1). The kinetic (A) and equilibrium (B) analyses were presented in Fig 2. The binding affinities of CBS with CAII were shown in Table 1. The results of this study were in agreement with previous reports [10, 11], revealing that SPR method is suitable for determining of the interaction between proteins and chemical compounds.

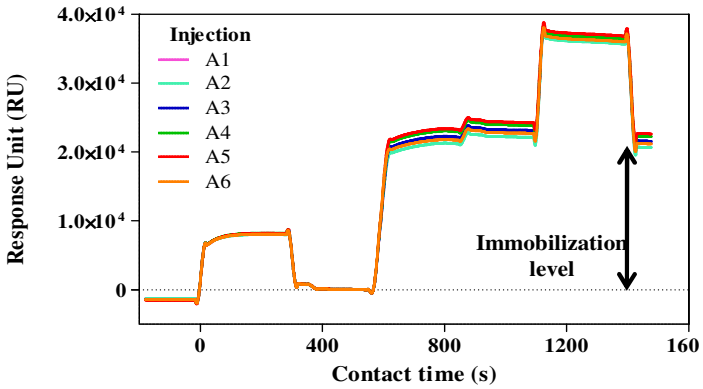

Fig. 1. Immobilization level of CALL on the SPR.

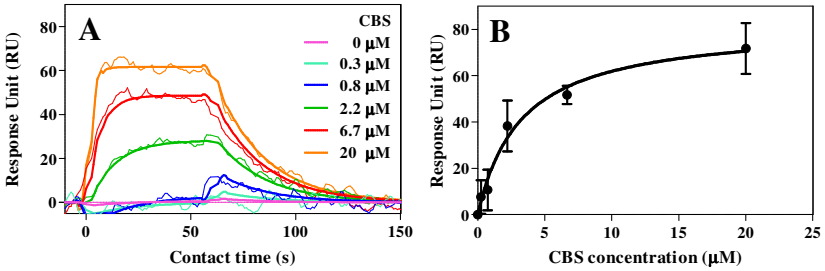

Fig. 2. Dose dependent response of CAII and CBS the interaction on SPR chip. *(A) Kinetic analysis: thicker lines represent a global fit of a simple interaction model to the experimental data (thin lines). (B) Equilibrium analysis: plot follows dose-dependent manner with the curves fit to a 1:1 equilibrium.*

3.2. Optimization of the interaction of hCAR with CITCO on SPR system

For amine coupling, a protein needs to dilute in a buffer that ensures a net positive charge on protein. Such a positively-charged protein will be attracted to the negatively charged surface of sensor chip. Thus, the buffer must be low ionic strength to minimize charge screening. The optimal pH of buffer can be predicted to be lower than the pI of protein one pH unit [12]. However, amine coupling is most efficient at high pH, because activated carboxylic groups react better with uncharged amino groups. Therefore, Na acetate buffer pH 5.0 that was approximately 1 unit lower than the pI of hCAR (6.24) was selected for hCAR dilution. The immobilization level of hCAR was 8.900 ± 240 RU (Fig. 3).

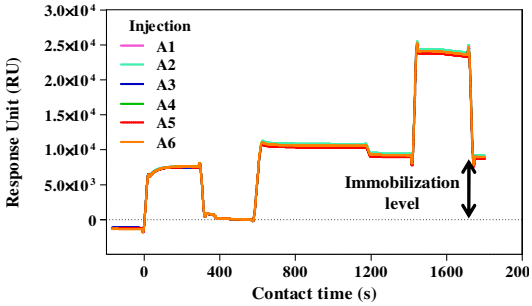

Fig. 3. Immobilization level of hCAR on the SPR.

CITCO, known as hCAR agonist [13] was used as positive control to develop the method for CAR-chemical interaction. To optimize the interaction of CITCO with hCAR, the maximum concentration of CITCO, the flow rate and contact time were modified as shown in the Table 2. The results of interaction between hCAR and CITCO were presented in the Table 2 and Fig. 4.

Table 2. Summary of interaction conditions and binding affinities of hCAR and CITCO

CITCO concentration (µM)	**Flow rate (µl/min)**	**Contact time (sec)**	**K_D (µM)**	**Note**
			His-hCAR LBD	**Corresponded Figures**
0, 12.5, 25, 50, 100, 200	100	60	21.2	Fig. 4-A2
0, 0.6, 1.9, 5.6, 17, 50	100	60	2.8 ± 1.1	Fig. 4-B2
0, 0.6, 1.9, 5.6, 17, 50	100	120	6.6 ± 1.2	Fig. 4-C2
0, 0.6, 1.9, 5.6, 17, 50	25	120	7.2 ± 2.2	Fig. 4-D2

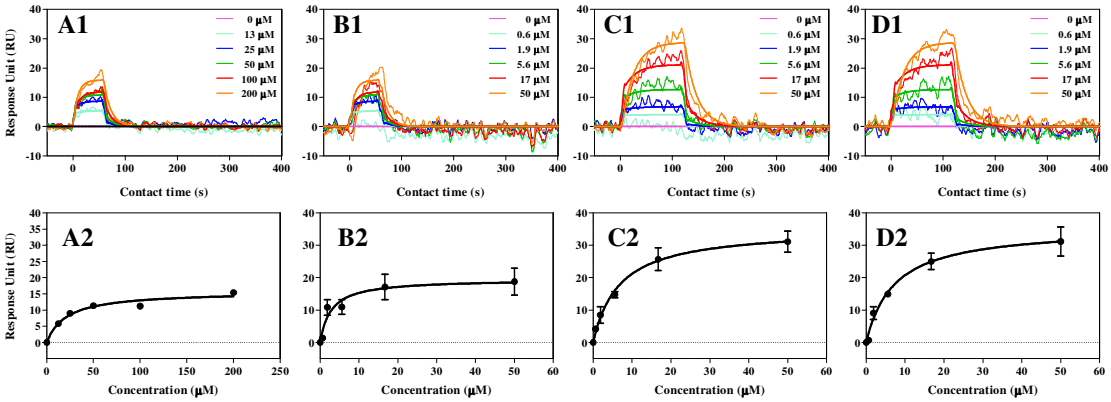

Fig. 4. Dose-dependent response in the interaction of hCAR with CITCO on SPR chip.
(1) - Kinetic analysis and (2) - Equilibrium analysis

The data showed that hCAR responses specifically with CITCO at all the experiments as expected. However, the response levels of hCAR with CITCO were different among modified conditions. The first analyzation of CITCO with the highest concentration was done with 200 μM. The five other concentrations were prepared by a twofold dilution series. The data showed the overlapping in the responses of hCAR at 50 and 100 μM of CITCO (Fig. 4-A1). Moreover, the response in the lowest concentration of CITCO was far from blank concentration in kinetic analysis. Equilibrium analysis also presented a 3-10 times higher K_D value (21.2 μM) than that of other tests. It means that this dilution series was not good for detect the binding affinity. Therefore, the highest concentration of CITCO was decreased to 50 μM and 5 other different concentrations were tested by three-fold dilutions in the next steps. The lowest K_D value (2.8 μM) in the 2nd test showed the strongest binding of hCAR with CITCO (Fig. 4-B1). However, the maximum response of hCAR with CITCO approximate 20RU was same as that of 1st test (Fig. 4-A1) and lower than those of 3rd and 4th tests with the contact time increased to 120s (Fig. 4-C1 and D1). These results showed that the longer time for interaction of hCAR and CITCO is necessary. To check whether the flow rate affects the interaction or not, the flow rate was decreased to 25 μl/min in the 4th test. In this condition, the responses of CITCO and hCAR were obvious (Fig. 4-D1) and similar with the response of 3rd test (Fig. 4-C1 and Table 2). With the lower flow rate, the requirement volume of CITCO for interaction is less to help save reagents. The results of this study revealed that the most effective conditions of CITCO on interaction with hCAR were at low flow rates and long contact time. This is in accordance with other interactions in which the reactors need time to interact with others. Although the specific binding of hCAR with CITCO was found as expected, but the K_D values (2,8-7 μM) in this assay were still higher than that in comparison with other assays (~49nM) [13]. The difference in these systems might be due to the distance from the experimental model. In our *in vitro* binding assay, we only used the LBDs of hCAR but other systems were conducted the interaction assay with the support by cofactor SRC1 [13].

4. Conclusion

This study showed the specific binding of hCAR and CITCO with equilibrium

dissociation constant (K_D) ranged from 2,8 to 7 μM. Among tested conditions, lower flow rates (25μl/min) and higher contact time (120s) appeared to be good conditions for detecting the specific binding affinity of CITCO with hCAR. The results revealed that the SPR-based biosensor system is an useful tool for screening the potential ligands of CAR as well as other proteins.

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Xác định tương tác của protein constitutive androstane receptor với CITCO bằng hệ thống biosensor trên nguyên lý cộng hưởng plasmon bề mặt

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Tóm tắt: Nghiên cứu này xác định ái lực gắn của protein CAR với CITCO (chất có khả năng gắn và hoạt hóa CAR từ người -hCAR) nhằm mục tiêu phát triển phương pháp sàng lọc nhanh các chất có tiềm năng gắn với các protein. Trước tiên, thiết bị SPR được kiểm tra khả năng ứng dụng bằng bộ kit chuẩn gồm protein CAII và chất gắn của nó CBS. Tiếp theo, protein đích được mã hóa từ vùng gen có khả năng gắn với ligand của hCAR sẽ được gắn cố định lên bề mặt của chip cảm biến SPR bằng tương tác của các nhóm amine. CITCO, chất hoạt hóa hCAR trong các nghiên cứu trước được sử dụng làm chất kiểm chứng dương để phát triển phương pháp xác định ái lực giữa hCAR đã gắn cố định trên chip cảm biến với các phân tử hóa chất. Như mong đợi, CITCO thể hiện tương tác đặc hiệu với protein hCAR trong nghiên cứu này. Kết quả cho thấy tiềm năng ứng dụng của hệ thống SPR trong việc sàng lọc các chất có tiềm năng gắn với protein CAR cũng như các protein khác.

Từ khóa: Constitutive androstane receptor, CITCO, thiết bị cộng hưởng plasmon bề mặt.