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Screening of chemokine receptor CCR4 antagonists by capillary zone electrophoresis

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KEYWORDS

Capillary zone electrophoresis;
CCR4 antagonist;
2-(2-(4-chloro-phenyl)-5-{[(naphthalen-1-ylmethyl)-carbamoyl]-methyl}-4-oxo-thiazolidin-3-yl)-N-(3-morpholin-4-yl-propyl)-acetamide;
Interactions;
Structural modification

Abstract CC chemokine receptor 4 (CCR4) is a kind of G-protein-coupled receptor, which plays a pivotal role in allergic inflammation. The interaction between 2-(2-(4-chloro-phenyl)-5-{[(naphthalen-1-ylmethyl)-carbamoyl]-methyl}-4-oxo-thiazolidin-3-yl)-N-(3-morpholin-4-yl-propyl)-acetamide (S009) and the N-terminal extracellular tail (ML40) of CCR4 has been validated to be high affinity by capillary zone electrophoresis (CZE). The S009 is a known CCR4 antagonist. Now, a series of new thiourea derivatives have been synthesized. Compared with positive control S009, they were screened using ML40 as target by CZE to find some new drugs for allergic inflammation diseases. The synthesized compounds XJH-5, XJH-4, XJH-17 and XJH-1 displayed the interaction with ML40, but XJH-9, XJH-10, XJH-11, XJH-12, XJH-13, XJH-14, XJH-3, XJH-8, XJH-6, XJH-7, XJH-15, XJH-16 and XJH-2 did not bind to ML40. Both qualification and quantification characterizations of the binding were determined. The affinity of the four compounds was valued by the binding constant, which was similar with the results of chemotactic experiments. The established CEZ method is capable of sensitive and fast screening for a series of lactam analogs in the drug discovery for allergic inflammation diseases.

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1. Introduction

Allergic inflammation diseases, such as asthma and atopic dermatitis, are Th2 lymphocyte-dominant inflammatory disease. CCR4 and its ligands play a role in Th2-type CD4+ T lymphocyte mediated inflammation [1–4]. CCR4 belongs to a family of CC chemokine receptors, which is a G-protein-coupled receptor with a characteristic seven-transmembrane structure. Current treatments for allergic inflammation include antihistamines and bronchodilators, which control symptoms but not disease progression. In addition, although corticosteroids specifically target the disease, its long-term use can cause

steroid resistance and side effects. Other safe and effective novel therapies for these diseases have been explored [5–7]. Several approaches are being developed to block the effects of chemokines, including small-molecule antagonists of chemokine receptors, modified chemokines and antibodies directed against chemokine receptors. Lactam analog, i.e. 2-(2-(2,4-dichloro-phenyl)-4-{[(2-methyl-3-chloro-phenyl)-1-ylmethyl]-carbamoyl}-methyl)-5-oxo-pyrrole-1-yl)-N-(3-piperidinyl-propyl)-acetamide (S009), as a novel antagonist of CCR4, displays high CCR4 binding affinity, excellent chemotaxis inhibitory activity and selectivity toward CCR4 [8]. Previous studies have been demonstrated that the amino termini of chemokine receptors are important in ligand-binding using the cell-based binding assays or multidimensional heteronuclear NMR spectroscopy, such as the interactions of CCR4 agonists, stromal cell-derived factor-1, eotaxin, interleukin-8, fractalkine and monocyte chemotactic protein-1 with the amino terminus of their cognate receptors, with high binding affinities, respectively [9–14]. Recently, we have reported that S009 as a potent antagonist of CCR4 has a high affinity with the N-terminal extracellular and both qualification and quantification characterizations of the binding were determined [15]. We envisaged that the basic pharmacophore for CCR4 binding of such compounds consisted of two wings attached to a central core in a defined configuration [9,16]. So our arrangement presented took place in these positions for favorable interactions. For these analogs, we screen them preliminarily using CZE first. Through this experiment, we hope to find other activity compounds and make preparations for further cell and animal experiments in the drug discovery for allergic inflammation diseases.

2. Experimental

2.1. Apparatus and CE conditions

All experiments were performed on a Beckman P/ACETM MDQ system (Beckman Coulter, Inc., Fullerton, CA, USA) equipped with a photodiode array detector as well as the 32 KaratTM software version 5.0 (Beckman). A capillary (Yongnian Optical Fibre Corp., Hebei, China) with an internal diameter of 75 μm was used. The total and effective lengths of the capillary were 30.2 cm and 10 cm, respectively. Before using, the new capillaries were rinsed with 0.1 M NaOH solution for 20 min, and subsequently with deionized water for 5 min.

2.2. Chemicals and materials

All chemicals were of analytical grade unless otherwise indicated. Tris base (ultrapure) and acetic acid used in this study were from Beijing Chemical Reagent Factory (Beijing, China). These analogs (Table 1), which are XJH-1, XJH-2, XJH-3, XJH-4, XJH-5, XJH-6, XJH-7, XJH-8, XJH-9, XJH-10, XJH-11, XJH-12, XJH-13, XJH-14, XJH-15, XJH-16 and XJH-17, were synthesized in Professor Song Li' laboratory (Laboratory of Computer-Aided Drug Design & Discovery, Beijing Institute of Pharmacology and Toxicology, 27 Taiping Road, Beijing 100850, China) and structure was identified by MS and NMR. It was dissolved in dimethyl sulfoxide and diluted to the concentration of 1 mM by phosphate buffered saline (PBS). The equivalent peptide derived from ML40 was synthetized from Chinese Peptide Company in Hangzhou, China, and subsequently purified (purity $\geq$95%) and characterized by reversed-phase high performance liquid chromatography and mass spectrometry. The synthetized amino acid sequence of the human CCR4 amino terminus was MNPTDIADTTLDESIYSNYYLYESIPKPCTKEGIKAFGEL. Deionized water was used to prepare buffer solutions and prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA). All buffers and solutions used in the study were filtered through 0.45 μm membranes (Agilent, Germany) before using.

2.3. Sample preparation

To investigate the interactions between the N-terminal extracellular tail of CCR4 and these analogs, different concentrations of the N-terminal extracellular tail of CCR4 (0 mM, 0.013 mM, 0.04 mM, 0.07 mM and 0.12 mM) were tested for the formation of complex, which was formed by mixing the analogs with the N-terminal extracellular tail of CCR4 in the running buffer. It was incubated for 20 min at 37 °C before CE analysis.

2.4. CZE conditions

To study the interactions between the N-terminal extracellular tail of CCR4 and these analogs, the temperatures of the cartridge and sample room were kept at 25 °C and 4 °C, respectively. Before each measurement, the capillary was rinsed with running buffer (30 mM Tris–HAc, pH 7.30). Samples containing the mixtures of the N-terminal extracellular tail of CCR4 and these analogs were injected using the pressure injection mode at 0.5 p.s.i. for 10 s (1 p.s.i. = 6894.76 Pa). The applied voltage was −10 kV. The capillary was washed between runs with the running buffer for 5 min at 20 p.s.i. Each concentration was run in duplicate.

2.5. Quantitative model for the binding study

In the binding studies, the binding constant and stoichiometry are important parameters to be determined. Scatchard analysis is a common way to linearize the binding data, as expressed in the following equation:

$$r/\mathrm{Cf} = -Kr + nK \quad (1)$$

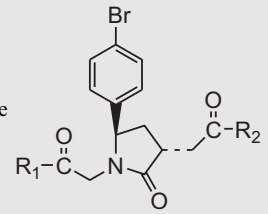
where r is the ratio of the concentrations of the bound ligand (or receptor) to the total receptor (or ligand) and Cf is the unbound ligand (or receptor) concentration. K is the apparent binding constant and n is the number of binding sites [17]. In this study, r is the concentration ratio of the bound compound A to the total standard N-terminal extracellular tail of CCR4 and Cf is the free compound A concentration.

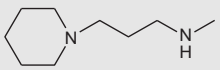
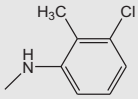
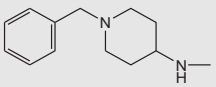
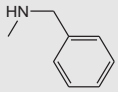
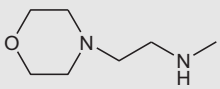
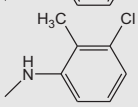
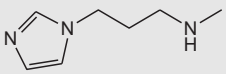
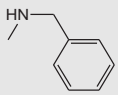
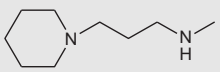
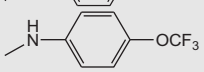
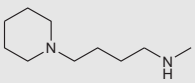
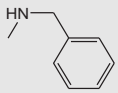
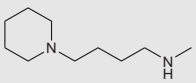
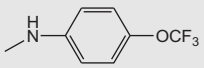
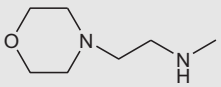
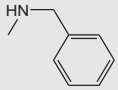
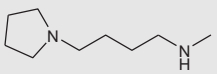
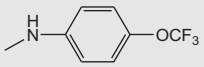
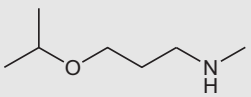
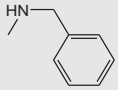
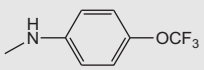
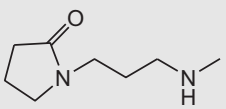
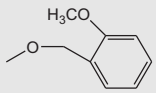
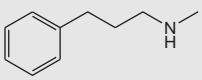
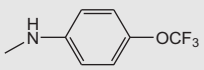
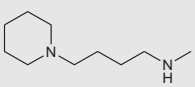
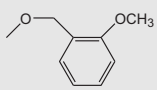
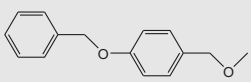
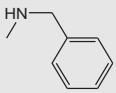
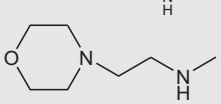
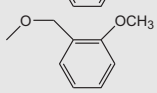
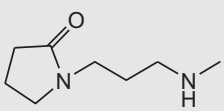
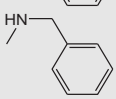
3. Results and discussion

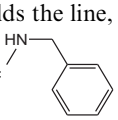
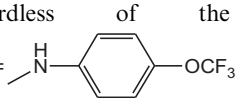
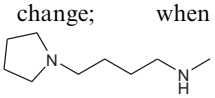
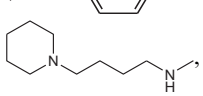
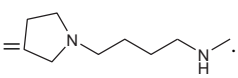
3.1. Preliminary screening for these compounds

In Fig. 1, while increasing the N-terminal extracellular tail concentration (0 mM, 0.013 mM, 0.04 mM, 0.07 mM and 0.12 mM), the variance of the peak height of these compounds was as the standard when the interaction happens. We can find that different compounds have the different response from electropherograms. So the ratio between each analogs and ML40 was different. We displayed the phenomenon of two of these analogs for model in this figure. The concentration of the

Table 1 Reorganization of the two wings and central core.

Center piece 

No.	Structure		No.	Structure	
	R_1	R_2		R_1	R_2
XJH-1			XJH-10		
XJH-2			XJH-11		
XJH-3			XJH-12		
XJH-4			XJH-13		
XJH-5			XJH-14		
XJH-6			XJH-15		
XJH-7			XJH-16		
XJH-8			XJH-17		
XJH-9					

N-terminal was according to the maximum concentration, which S009 interacted with ML40 in the previous report [18]. Compared with positive control S009, XJH-9, XJH-10, XJH-11, XJH-12, XJH-13, XJH-14, XJH-3, XJH-8, XJH-6, XJH-7, XJH-15, XJH-16 and XJH-2 did not bind to ML40; XJH-5, XJH-4, XJH-17 and XJH-1 displayed the interaction with ML40. This result shows that if the central core holds the line, compound does not interact with ML40 when R2=  regardless of the R1's change; when R2=  and R1=  and  , the interaction between compounds and ML40 happened and especially, the peak height is decreased sharply with R1=  . When the structure was ester, only the compound with R_1= has the interaction with ML40. In the series of R2= , there was no interaction taking place only when the central core changed into .

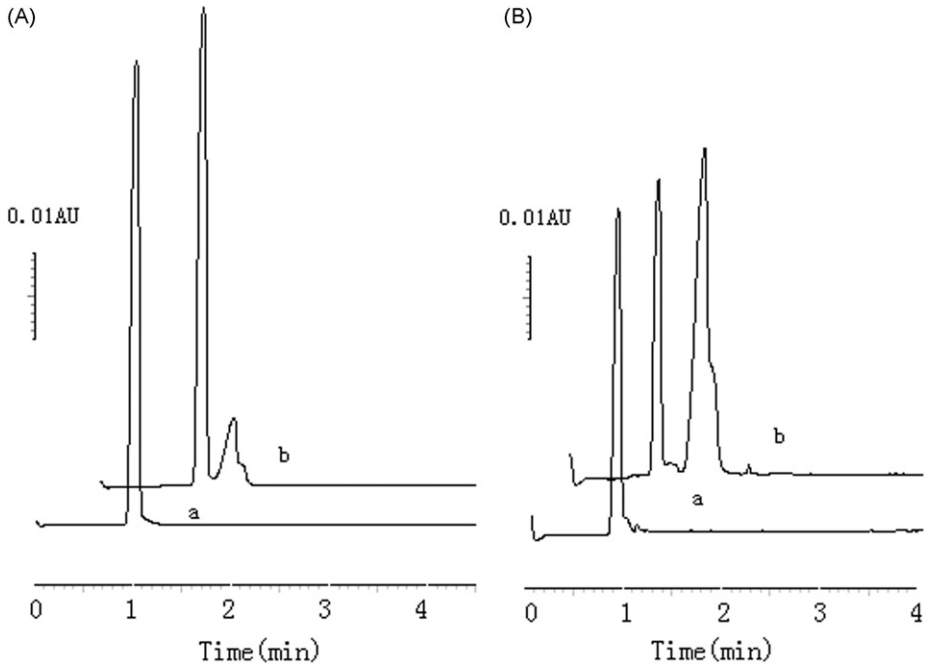


Figure 1 Electropherograms for compounds screening containing a fixed concentration of the compound at 0.67 mM (a) and fixed concentrations of ML40 at 0.12 mM (b). The conditions used were same as follows: Beckman P/ACE MDQ capillary electrophoresis system. Injection: 0.5 p.s.i. for 10 s. Applied voltage: −10 kV. Capillary: capillary of 30.2 cm (effective length 10 cm) 75 μm I.D. Running buffer: 30 mM Tris–HAc, pH 7.3. The different compounds were (A) XJH-9; (B) XJH-10; the electropherograms of other compounds were similar with the above.

Table 2 Relationship between peak height and these analogs' concentration.

Compounds	Regression equations	Correlation coefficient	Concentrations (mM)
XJH-5	$y=43537x-6606.3$	0.9775	0.22–0.50
XJH-4	$y=319553x-20126$	0.9978	0.10–0.20
XJH-17	$y=107760x-5162.6$	0.9831	0.20–0.50
XJH-1	$y=125820x+2797.8$	0.9600	0.29–1.00

The experimental results can provide some evidence for structural modification and structure–activity relationship.

3.2. Interactions between the positivity compound and the N-terminal extracellular tail of CCR4

To obtain a calibration plot, increasing concentrations of these analogs were injected into the capillary, the peak heights being proportional to the concentrations. The relationship between peak height and these analogs concentration is expressed in Table 2. The corresponding concentrations of the compounds in the binding study were calculated from this calibration plot. Fig. 2 shows electropherograms for sample alone and mixtures. By increasing the N-terminal extracellular tail concentration the peak height of the compounds is decreased, indicating strong binding. Theoretically, the dissociation rate of the complex is small and consequently it could be detected.

The binding constant is an important quantitative parameter for characterizing the interaction between the N-terminal extracellular tail of CCR4 and the compounds. The concentrations of the free compounds were calculated from the calibration plot to obtain the values of r and from the slope of the Scatchard plot (Table 3), K was calculated to be XJH-5: $(1.69\pm0.11)\times10^5\ M^{-1}$; XJH-4: $(3.51\pm0.93)\times10^4\ M^{-1}$; XJH-1: $(1.60\pm0.04)\times10^4\ M^{-1}$; XJH-17: $(4.94\pm0.34)\times10^4\ M^{-1}$ (Table 3). The result shows that XJH-5 has the strongest affinity with the N-terminal extracellular tail and the other three compounds also have high affinity with it. Compared with the compound action of chemotaxis inhibition potency and its binding constant with the N-terminal extracellular tail of CCR4 determined by CZE, we found that the compounds with higher affinity had stronger action of chemotaxis inhibition. An attractive direction is to apply CE to high-throughput drug screening for biological activity [19,20]. With the aid of CE, binding constants and stoichiometry data can be obtained while conventional drug screening techniques can only provide the IC50 values. Our experiment shows that CE can be employed as one of the reliable screening methods to a series of lactam analogs in the drug discovery for allergic inflammation diseases and CE can be employed to help analyze the structure–activity relationship of compounds.

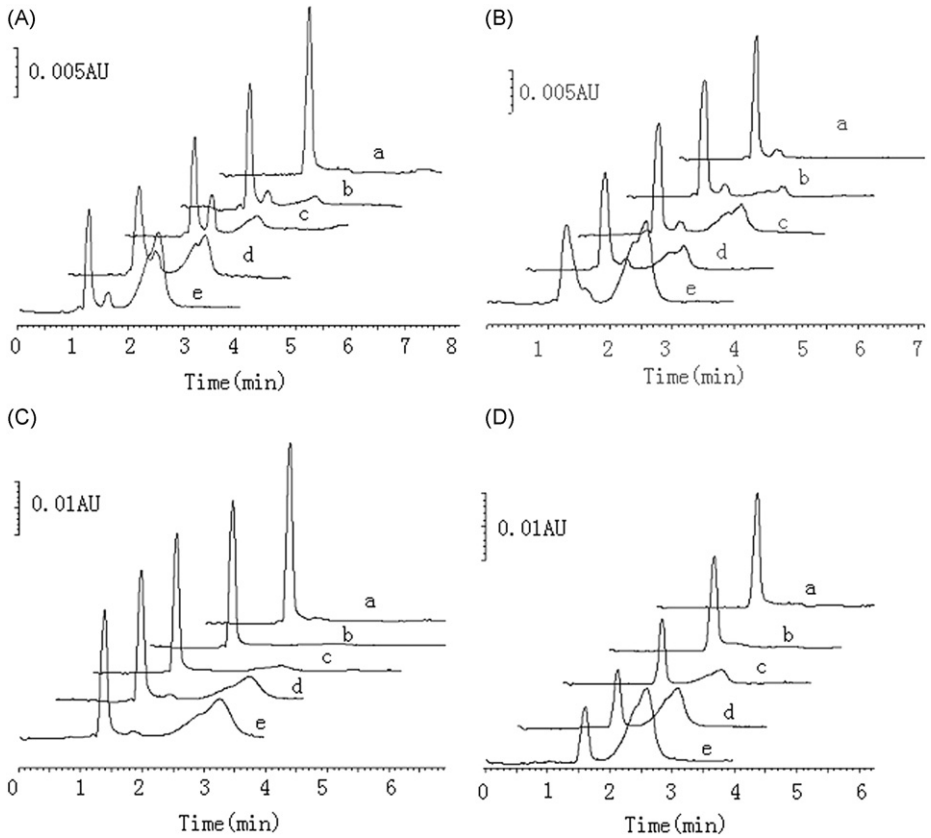


Figure 2 Electropherograms for four samples alone and mixtures containing a fixed concentration of the compound at 0.67 mM and increasing concentrations of ML40. Various concentrations of ML40: (a) 0 mM; (b) 0.013 mM; (c) 0.04 mM; (d) 0.07 mM; (e) 0.12 mM. The conditions used were the same as Fig. 1. The different interactioned compounds were (A) XJH-5; (B) XJH-4; (C) XJH-1; (D) XJH-17.

Table 3 Compounds and the binding constants.

Compounds	Regression equations	Correlation coefficient	Binding constants ($\times 10^4\ M^{-1}$)	RSD
XJH-5	$y=-14.464x+4.8339$	0.7765	16.9	0.11
XJH-4	$y=-5.8123x+0.6026$	0.9795	3.51	0.93
XJH-1	$y=-1.6313x+0.4412$	0.8006	1.60	0.04
XJH-17	$y=-4.6579x+2.2068$	0.7621	4.94	0.34

4. Conclusions

A series of analogs have been synthesized. Compared with positive control S009, we screen them targeting at ML40 using CZE for finding some new and further cell and animal experiments in the drug discovery for allergic inflammation diseases. XJH-9, XJH-10, XJH-11, XJH-12, XJH-13, XJH-14, XJH-3, XJH-8, XJH-6, XJH-7, XJH-15, XJH-16 and XJH-2 did not bind to ML40; XJH-5, XJH-4, XJH-17 and XJH-1 displayed the interaction with ML40. Both qualification and quantification characterizations of the binding were determined. The K values of four samples were calculated to be XJH-5: $(1.69\pm0.11)\times10^5\ M^{-1}$; XJH-4: $(3.51\pm0.93)\times10^4\ M^{-1}$; XJH^{-1}: $(1.60\pm0.04)\times10^4\ M^{-1}$; XJH-17: $(4.94\pm0.34)\times10^4\ M^{-1}$ by Scatchard analysis. The affinity of the four compounds was valued by the binding constant, which was similar with the biological experiment. Each run was completed within 4 min. The CZE provides a highly efficient, fast, quantitative and sensitive method for screening a series of lactam analogs in the drug discovery for allergic inflammation diseases. Also, our result can provide some evidence for structure–activity relationship.

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