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# Relationship between the structure and immunogenicity of ceftriaxone

**Arjharn W[1], Vongsutilers V[1,3], Thantiworasit P[2], Suthumchai N[2], Klaewsongkram J[2**], Tuesuwan B[1,3*]**

*[1] Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*

*[2] Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*

*[3] Medicinal Analytical and Pharmaceutical Chemistry Research Unit (MAPCU), Chulalongkorn University Drug and Health Products Innovation Promotion Center (CU.D.HIP), Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*

** E-mail: btuesuwan@pharm.chula.ac.th; ** E-mail: jettanong.k@chula.ac.th*

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## Introduction

Ceftriaxone is one of the most reported cause allergic reaction due to frequently prescribed.[1] Once the patients are suspected to ceftriaxone allergy, we must be concerned with cross-allergic reaction to cephalosporins and other β-lactam antibiotics. The relationship between structure and immunogenicity of β-lactam antibiotics could be beneficial for drug treatment selection. Several studies have attempted to reveal the structure of cephalosporins that involved the allergic reaction and cross reaction to other β-lactam antibiotics by skin test, re-challenge test, and radioimmunoassay on IgE determination.[2-6] These methods have disadvantages such as invasion or limitation of time interval from allergen exposure.[7] The in vitro immunoassay for instance enzyme-linked immunosorbent spot (ELISPOT) is an optional method to investigate the allergic reaction due to specificity and non-invasive.[8] Our objective is to investigate the relationship between structure of ceftriaxone and its immunogenicity using ELISPOT IFN-γ assay. The tested compounds are including native molecule of ceftriaxone, its side chain moieties, degradation products, and other native cephalosporins.

## Materials and methods

Chemicals and reagents: Ceftriaxone disodium hemiheptahydrate, cefotaxime sodium, ceftazidime, and 7-aminodesacetoxycephalosporanic acid (7ADCA) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Cefoperazone and cefepime hydrochloride were purchased from S.Z. Phystandard Bio-Tech Co., Ltd. (Guangdong, China). Cefpirome sulfate was purchased from BePharm Ltd. (Shanghai, China). 2-Amino-α-(methoxyimino)-4-thiazoleacetic acid, and tetrahydro-2-methyl-3-thioxo-1,2,4-triazine-5,6-dione were purchased from Sigma-Aldrich (Wisconsin, USA). All other chemicals were at least analytical grade.

**Tested compounds**: Tested compounds, including native molecule of ceftriaxone, side chain moieties, degradation products, and other native cephalosporins, were shown in Figure 1. All of compounds were confirmed by NMR spectroscopy and mass spectrometry techniques. $^{1}$H NMR and $^{13}$C NMR spectra were recorded on Bruker AVANCE III HD spectrometer at 500 MHz. High-resolution mass spectra (HRMS) were recorded on Bruker micrOTOF-QII mass spectrometer with an electrospray ionization ion source (ESI-Q-TOF). Purity of tested compounds (>95%) were determined by using high performance liquid chromatography coupled to diode array detector (HPLC/DAD).

**Ceftriaxone**: $^{1}$H NMR (500 MHz, $D_2O$): δ 3.36 (d, *J*=18Hz, 1H), 3.50 (s, 3H), 3.62 (d, *J*=18Hz, 1H), 3.85 (s, 3H), 3.94 (d, *J*=13.5Hz, 1H), 4.23 (d, *J*=13.5Hz, 1H), 5.08 (d, *J*=4.5Hz, 1H), 5.66 (d, *J*=5Hz,1H), 6.89 (s, 1H). $^{13}$C NMR (500 MHz, $D_2O$): δ 26.53, 33.68, 42.68, 57.19, 58.68, 62.67, 113.40, 119.17, 130.33, 140.39, 148.05, 156.71, 160.59, 163.66, 164.37, 164.86, 168.59, 170.91. Formula: $C_{18}H_{18}N_8O_7S_3$. HRMS (ESI): *m/z* 555.0531 ([M+H]$^+$). Purity: 98.1% (HPLC/DAD).

**Cefotaxime**: $^{1}$H NMR (500 MHz, $D_2O$): δ 1.99 (s, 3H), 3.29 (d, *J*=18Hz, 1H), 3.56 (d, *J*=18Hz, 1H), 3.88 (s, 3H), 4.61 (d, *J*=12.5Hz, 1H), 4.78 (d, *J*=12.5Hz, 1H), 5.10 (d, *J*=5Hz, 1H), 5.71 (d, *J*=4.5Hz, 1H), 6.90 (s, 1H). $^{13}$C NMR (500 MHz, $D_2O$): δ 20.31, 25.68, 57.19, 58.82, 62.73, 64.17, 113.26, 116.33, 131.51, 140.00, 147.78, 163.84, 164.69, 168.40, 170.88, 174.10. Formula: $C_{16}H_{17}N_5O_7S_2$. HRMS (ESI): *m/z* 456.0653 ([M+H]$^+$). Purity: 100.0% (HPLC/DAD).

**Cefepime**: $^{1}$H NMR (500 MHz, DMSO-*d6*): δ 2.03-2.10 (m, 2H), 2.03-2.10 (m, 2H), 2.93 (s, 3H), 3.41-3.44 (m, 2H), 3.58-3.63 (m, 2H), 3.66 (d, *J*=17Hz, 1H), 3.91 (s, 3H), 4.04 (d, *J*=17.5Hz, 1H), 4.33 (d, *J*=13.5Hz, 1H), 4.59 (d, *J*=13.5Hz, 1H), 5.33 (d, *J*=5Hz, 1H), 5.87 (dd, *J*=5, 8Hz, 1H), 6.88 (s, 1H), 9.83 (d, *J*=8Hz, 1H). $^{13}$C NMR (500 MHz, DMSO-*d6*): δ 20.57, 21.11, 28.57, 47.21, 58.41, 59.01, 62.74, 63.01, 63.69, 64.19, 110.18, 113.32, 132.78, 145.38, 161.14, 162.95, 163.32, 169.76. Formula: $C_{19}H_{25}N_6O_5S_2$. HRMS (ESI): *m/z* 481.1334 ([M]$^+$). Purity: 100.0% (HPLC/DAD).

**Cefpirome**: $^{1}$H NMR (500 MHz, DMSO-*d6*): δ 1.79-1.95 (m, 2H), 2.97-3.10 (m, 2H), 3.37 (s, 2H), 3.81 (s, 3H), 5.18 (d, *J*=5Hz, 1H), 5.43 (d, *J*=15.5Hz, 1H), 5.65 (d, *J*=15.5Hz, 1H), 5.85 (dd, *J*=5, 8Hz, 1H), 6.72 (s, 1H), 7.27 (s, 2H), 7.92 (t, *J*=7Hz, 1H), 8.34 (d, *J*=7.5Hz, 1H), 8.73 (d, *J*=6Hz, 1H), 9.64 (d, *J*=8Hz, 1H). $^{13}$C NMR (500 MHz, DMSO-*d6*): δ 19.91, 21.03, 25.08, 26.43, 28.22, 57.51, 58.89, 61.93, 108.96, 119.23, 124.35, 128.66, 139.31, 142.06, 143.29, 145.95, 148.74, 154.70, 162.82, 162.88, 163.71, 168.47. Formula: $C_{22}H_{23}N_6O_5S_2$. HRMS (ESI): *m/z* 515.1165 ([M]$^+$). Purity: 100.0% (HPLC/DAD).

**Ceftazidime**: $^{1}$H NMR (500 MHz, DMSO-*d6*): δ 1.38 (s, 3H), 1.39 (s, 3H), 3.10 (d, *J*=17.5Hz, 1H), 3.51 (d, *J*=17.5Hz, 2H), 5.07 (d, *J*=4.5Hz, 1H), 5.20 (d, *J*=13.5Hz, 1H), 5.65 (d, *J*=13Hz, 1H), 5.72 (dd, *J*=5, 7.5Hz, 1H), 6.68 (s, 1H), 7.25 (s, 2H), 8.12 (t, *J*=7Hz, 2H), 8.55 (t, *J*=7.8Hz, 1H), 9.41 (d, *J*=6Hz, 2H), 9.81 (s, 1H). $^{13}$C NMR (500 MHz, DMSO-*d6*): δ 23.96, 24.36, 57.45, 58.54, 61.52, 82.13, 109.37, 109.72, 128.03, 138.08, 142.76, 145.08, 145.64, 149.37, 162.60, 163.14, 163.19, 168.46, 175.44. Formula: $C_{22}H_{23}N_6O_7S_2$. HRMS (ESI): *m/z* 547.1072 ([M]$^+$). Purity: 100.0% (HPLC/DAD).

**Cefoperazone**: $^{1}$H NMR (500 MHz, DMSO-*d6*): δ 1.07 (t, *J*=7Hz, 3H), 3.51-3.55 (m, 4H), 3.65 (d, *J*=18Hz, 2H), 3.87-3.91 (m, 2H), 3.92 (s, 3H), 4.18-4.32 (dd, *J*=13.5, 56.5Hz, 2H), 5.98 (d, *J*=4.5Hz, 1H), 5.47 (d, *J*=7.5Hz, 1H), 5.71 (dd, *J*=4.5, 8Hz, 1H), 6.71 (d, *J*=9Hz, 2H), 7.20 (d, *J*=8.5Hz, 2H), 9.33 (d, *J*=9.5Hz, 1H), 9.44 (s, 1H), 9.70 (d, *J*=7.5Hz, 1H). $^{13}$C NMR (500 MHz, DMSO-*d6*): δ 11.91, 26.78, 33.76, 35.45, 41.63, 42.81, 56.34, 57.48, 58.54, 115.20, 125.30, 125.77, 127.96, 128.07, 151.83, 153.07, 155.38, 157.12, 159.46, 162.75, 164.20, 170.45. Formula: $C_{25}H_{27}N_9O_8S_2$. HRMS (ESI): *m/z* 646.1504 ([M+H]$^+$). Purity: 100.0% (HPLC/DAD).

**7ADCA**: $^{1}$H NMR (500 MHz, DMSO-*d6*): δ 1.96 (s, 3H), 3.54 (d, *J*=18Hz, 1H), 3.54 (d, *J*=18Hz, 1H), 4.68 (d, *J*=5Hz, 1H), 4.90 (d, *J*=5Hz, 1H). $^{13}$C NMR (500 MHz, DMSO-*d6*): δ 19.36, 28.39, 58.49, 63.30, 122.82, 127.78, 163.81, 169.52. Formula: $C_8H_{10}N_2O_3S$. HRMS (ESI): *m/z* 215.0485 ([M+H]$^+$). Purity: 100.0% (HPLC/DAD).

**2-Amino-α-(methoxyimino)-4-thiazoleacetic acid**: $^{1}$H NMR (500 MHz, DMSO-*d6*): δ 3.84 (s, 3H), 6.84 (s, 1H), 7.24 (s, 2H). $^{13}$C NMR (500 MHz, DMSO-*d6*): δ 62.25, 108.32, 141.41, 148.19, 163.86, 168.78. Formula: $C_6H_7N_3O_3S$. HRMS (ESI): *m/z* 202.0286 ([M+H]$^+$). Purity: 100.0% (HPLC/DAD).

**Tetrahydro-2-methyl-3-thioxo-1,2,4-triazine-5,6-dione**: $^{1}$H NMR (500 MHz, DMSO-*d6*): δ 3.63 (s, 3H), 12.42 (s, 1H), 13.00 (s, 1H). $^{13}$C NMR (500 MHz, DMSO-d6): δ 44.18, 150.15, 152.59, 169.83. Formula: $C_4H_5N_3O_2S$. HRMS (ESI): *m/z* 160.0185 ([M+H]$^+$). Purity: 100.0%(HPLC/DAD).

**Synthesis of desacetylcefotaxime**: Cefotaxime sodium (0.936 g) was dissolved in 20 mL of 0.3 N NaOH. The reaction solution was stirred in ice-bath with temperature not greater than 5ºC. After 3 hours, the concentrated HCl was added into the stirred solution to bring pH down to 7-8. The reaction solution was cleaned up using solid-phase extraction (SPE) with octadecyl (C18) cartridge and dried by freeze-dry technique. Yield: 53.8%. $^{1}$H NMR (500 MHz, $D_2O$): δ 3.36 (d, *J*=18Hz, 1H), 3.56 (d, *J*=17.5Hz, 1H), 3.88 (s, 3H), 4.16 (dd, 2H), 5.10 (d, *J*=4.5Hz, 1H), 5.68 (d, *J*=4.5Hz, 1H), 6.91 (s, 1H). $^{13}$C NMR (500 MHz, $D_2O$): δ 23.08, 25.58, 57.21, 58.67, 61.01, 62.68, 113.44, 121.28, 129.50, 140.36, 148.08, 163.88, 164.92, 169.01. Formula: $C_{14}H_{15}N_5O_6S_2$. HRMS (ESI): *m/z* 414.0447 ([M+H]$^+$). Purity: 96.5% (HPLC/DAD).

**Synthesis of desacetylcefotaxime lactone**: Cefotaxime sodium (0.468 g) was dissolved in 10 mL of 0.3 N NaOH. The reaction solution was stirred in ice-bath with temperature not greater than 5ºC. After 3 hours, the concentrated HCl was added into the stirred solution to bring pH down to about 2. The reaction was brought to proceed by stir at room temperature. After 2 hours, the stirred solution was cooled down in ice-bath and brought pH up to 7-8 using 2 N NaOH. The formed precipitate was collected using filter paper No.1 and washed with small amount of cold ultrapure water. The moist precipitate was dried by freeze-dry technique. Yield: 60.5%. $^{1}$H NMR (500 MHz, DMSO-*d6*): δ 3.78 (d, *J*=7Hz, 2H), 3.84 (s, 3H), 5.04 (s, 2H), 5.15 (d, *J*=5Hz, 1H), 5.92 (dd, *J*=5, 8Hz, 1H), 6.74 (s, 1H), 7.25 (s, 2H), 9.67 (d, *J*=8Hz, 1H). $^{13}$C NMR (500 MHz, DMSO-*d6*): δ 22.54, 57.37, 59.20, 61.88, 71.36, 108.79, 122.73, 142.28, 142.47, 148.86, 162.89, 163.05, 166.45, 168.39. Formula: $C_{14}H_{13}N_5O_5S_2$. HRMS (ESI): *m/z* 396.0431 ([M+H]$^+$). Purity: 97.4% (HPLC/DAD).

**Biological samples**: The control samples, peripheral blood mononuclear cells (PBMC), were obtained from healthy volunteers (3 males and 7 females, 29-43 years) without a history of β-lactam antibiotics allergy. Whole blood was collected into acid-citrate dextrose (ACD) anticoagulant tube. The PBMC was separated from whole blood by Ficoll-Hypaque density gradient centrifugation. The research protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 131/58, Approved date: September 17, 2015). The allergic samples, PBMC that obtained from patients (1 males and 5 females, 24-75 years), with history of ceftriaxone and other β-lactam antibiotics (cefuroxime and meropenem) allergy, the retained samples, were obtained from study with title of "In vitro investigation to study cross-reactivity reactions to third generation cephalosporins in patients with a history of beta-lactam allergy" (IRB No. 251/57, Approved date: August 7, 2014).

**Table 1**. Result of ELISPOT IFN-γ assay

| PBMC ID / Amount (cells per well) | History of allergic drug | ELISPOT IFN-γ assay with tested compound | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Native compounds (3rd,4th generation cephalosporins) | | | | | | Bicyclic β-lactam core and side chains | | | Degraded products | |
| | | CTX | CFT | CFP | CPR | CTZ | CFZ | 7ADCA | MTTA | TMTD | DAC | DAL |
| Group A (n=4) | | | | | | | | | | | | |
| P01/0.5x10$^5$ | Ceftriaxone | - | - | - | - | - | - | + | - | - | - | + |
| P02/0.5x10$^5$ | Ceftriaxone | - | + | - | + | - | + | + | - | - | + | - |
| P03/0.8x10$^5$ | Ceftriaxone | - | - | - | - | - | - | - | - | - | + | - |
| P04/1.0x10$^5$ | Ceftriaxone | + | + | - | + | - | - | + | - | - | + | - |
| Group B (n=2) | | | | | | | | | | | | |
| P05/0.8x10$^5$ | Cefuroxime | - | - | - | - | - | - | - | - | - | - | - |
| P06/0.8x10$^5$ | Meropenem | - | - | - | - | - | - | - | - | - | - | - |

"Group A" = PBMC obtained from patients with history of ceftriaxone allergy; "Group B" = PBMC obtained from patients with history of cefuroxime or meropenem allergy; "+" = Positive response; "-" = Negative response

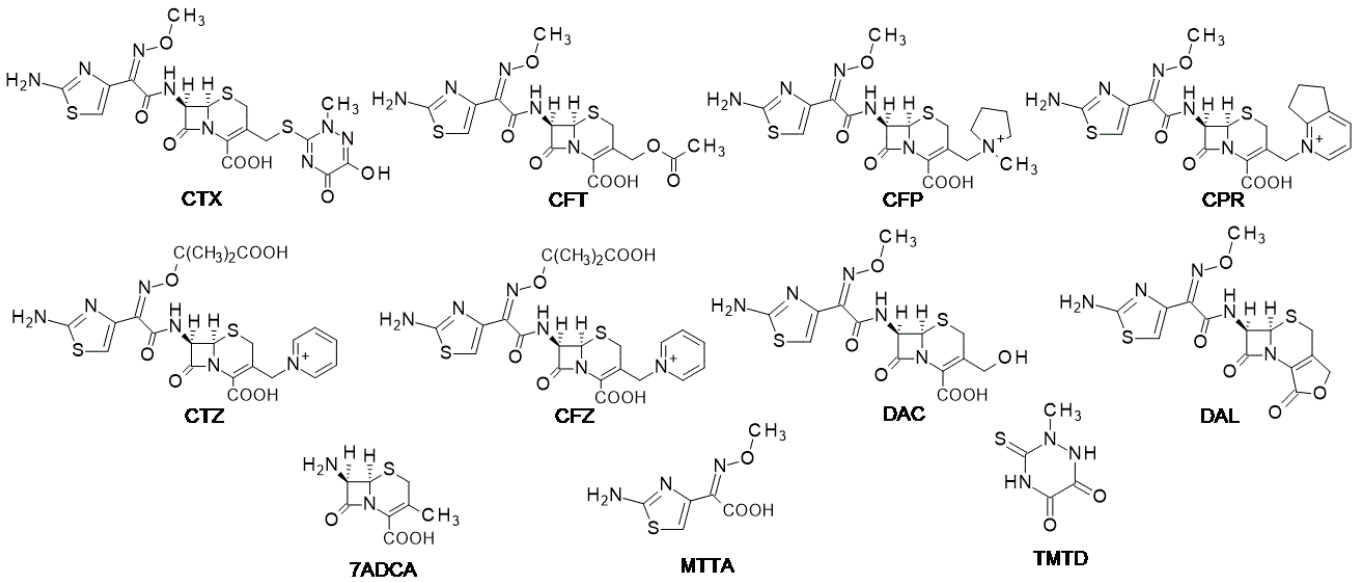


**Figure 1**. Tested compounds were consisted of; Ceftriaxone (CTX); Cefotaxime (CFT); Cefepime (CFP); Cefpirome (CPR); Ceftazidime (CTZ); Cefoperazone (CFZ); Desacetylcefotaxime (DAC); Desacetylcefotaxime lactone (DAL); 7-Aminodesacetoxycephalosporanic acid (7ADCA); 2-Amino-α-(methoxyimino)-4-thiazoleacetic acid (MTTA); Tetrahydro-2-methyl-3-thioxo-1,2,4-triazine-5,6-dione (TMTD).

**ELISPOT assay:** The procedure of ELISPOT assay was modified from Tanvarasethee B *et al*.[9] The number of IFN-γ releasing cells were determined after incubated with tested compounds (5-200 μg/mL). Result was expressed as the numbers of IFN-γ SFC/$10^6$ PBMC cultured with the tested compound, subtracted by the values obtained from PBMC cultured without the tested compound. Here, the numbers of spot greater than the mean plus 2 standard deviations of the spot from 10 healthy volunteers, each tested compound, were defined as positive.

**Results**

The result from ELISPOT IFN-γ assay was shown in Table 1. We found positive responses to tested compounds in PBMC from patients with history of ceftriaxone allergy (group A). The 7ADCA and DAC showed three positive responses, CFT and CPR each showed two, while CTX, CFZ and DAL showed one. On the other hands, PBMC obtained from patients with history of cefuroxime or meropenem allergy (group B) exhibited negative response to all tested compounds.

**Discussion**

For our study, we found no positive response of ELISPOT IFN-γ assay in PBMC neither from patients with history of cefuroxime allergy nor meropenem allergy to all tested compounds. DAC which could be derived from hydrolysis of CTX, CFT, CFP, or CPR exhibited high ability to stimulate the IFN-γ secretion from PBCM of the group A. The result suggested that DAC can involve the allergic reaction of CTX.

TMTD (side chain at C3 of CTX) and MTTA (side chain at C7 of CTX) did not elicited positive IFN-γ secretion of PBMC from one patient in group A, whereas DAC, a bicyclic β-lactam core together with side chain at C7 of CTX, elicited three positive responses. The result implied that DAC could play a role in allergic reaction more than a side chain alone, MTTA. PBMC from four patients were tested with CTX and only one showed positive response despite using PBMC obtained from patient all with history of CTX allergy. When these PBMC from four patients were tested with DAC, we found three positive responses. This clearly indicated DAC is of value in allergic screening for CTX. DAC, in particular, seems good starting candidate for further investigation for screening of CTX hypersensitivity.

CFT and CPR, are shared the same bicyclic β-lactam core and side chain with CTX, presented two positive responses in PBMC from group A. The positive results of CFT and CPR might be mainly caused by bicyclic β-lactam core together with side chain that gave an appropriate conformation to contact with PBMC and stimulate the IFN-γ secretion.

However, 7ADCA also showed three positive responses. Despite shared bicyclic β-lactam core in cephalosporins, CFZ showed only one positive response in PBMC from a patient with history of CTX allergy while CFP and CTZ were not. It need more investigation in the future.

With only one out four positive ELISPOT assay for CTX, we must consider possibility of amount and deterioration of PBMC employed in this study. Whereas lacked of PBMC from CTX allergic patients, therefore it was necessary to use in amount of 0.5-1.0x$10^5$ cells per well with lower than in case of PBMC from healthy volunteers, 1.5-2.5x$10^5$ cells per well. However, tested compound, especially DAC, was able to present positive responses in PBMC from CTX allergic patients while was not in cefuroxime nor meropenem allergic patients. Despite possibility of PBMC deterioration, DAC still yielded three positive results which is far better than CTX. This clearly indicates DAC can initiate specific immunological reaction since we found no false positive responses of ELISPOT IFN-γ assay in patients with history of cefuroxime and meropenem allergy to all tested compounds. Future study could eventually lead to development of standard testing kit.

## Conclusion

In conclusion, we study the relationship between structure and immunogenicity of ceftriaxone used varieties of tested compounds that including molecule of ceftriaxone, side chain moieties of ceftriaxone, bicyclic β-lactam core, degraded products, cefotaxime, cefepime, cefpirome, ceftazidime, and cefoperazone. All of tested compounds were confirmed and determined the purity prior to use. The immunogenicity of tested compounds were evaluated using ELISPOT IFN-γ assay to compare between ceftriaxone, cefuroxime and meropenem allergic patients. According to our data, the results indicated that the 7ADCA and desacetylcefotaxime can initiate specific immunological reaction in PBCM from ceftriaxone allergic patients. Future study could eventually lead to development of standard testing kit.

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