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Inhibition of Human Neutrophil Elastase by Tetracyclines and Mechanism of the Inhibition*

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ABSTRACT

Human neutrophil elastase (HNE, EC 3, 4, 21, 11), a mediator of tissue breakdown, was inhibited by tetracycline, oxytetracycline and demeclocycline. Among them, oxytetracycline showed the most potent inhibitory effect on the activity of HNE. IC50 of this drug at our specific condition was less than 1 mM. Tetracycline inhibited human neutrophil elastase non-competitively, and oxytetracycline inhibited competitively. Ki values of tetracycline and oxytetracycline were 4.9 mM and 0.39 mM, respectively.

Structural modified tetracycline, de-dimethylaminotetracycline, which showed no antibiotic activity since the active dimethylamino radical was removed from the position #4 of the tetracycline, showed similar inhibition effect on the activity of human neutrophil elastase to that of tetracycline. Thus, we speculated that inhibition of human neutrophil elastase by tetracyclines was not depended on the dimethylamino radical which is a critical active site for antibiotic effect, rather it was depended on the hydoxyl radical of tetracyclines. Therefore, the property of inhibiting elastase may be an additional molecular biochemical mechanism of action of these drugs at the inflammatory sites.

Key Words: Neutrophil elastase, Tetracycline, Oxytetracycline, De-dimethylaminotetracycline

INTRODUCTION

Human neutrophil elastase (HNE, EC 3, 4, 21, 11), which has an antimicrobial action against microorganisms (Elsbach *et al.*, 1985) are usually regulated its enzymatic activity by plasma proteinase inhibitors, alpha-1-proteinase inhibitor and alpha-2-macroglobulin (Janoff, 1972c; Cohen, 1975; Starky, 1977). Under certain pathological conditions, however, over released enzyme or abnormal function of inhibitors may cause the various inflammatory diseases (Janoff, 1972a; Fritz *et al.*, 1986) including emphysema (Mittan, 1972; Goldston *et al.*, 1973; Cohen, 1983) and rheumatoid arthritis (Glynn, 1972).

Recently, we have reported that antibiotics including cefamandole, oxytetracycline, methicillin effectively inhibited the activity of HNE (Ghim *et al.*, 1989). From this result, we speculated that some antibiotics which showed inhibition effect on the activity of HNE possibly have an additional mechanism of action of those specific drugs at the inflammatory sites, beside of the known mechanism of action as a antimicrobial agents. And this newly discovered property of the drugs could provide a novel approach to the treatment of some inflammatory diseases caused by HNE. Therefore, this present report describes

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the possible mechanisms of inhibition effect of tetracyclines on the activity of HNE.

MATERIALS AND METHODS

Materials

N-Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (SAPNA), N-Succinyl-Ala-Ala-Ala-p-Nitroanilide (SANA), Oxytetracycline, Tetracycline, Demeclocycline were purchased from Sigma chemical Co., Ultrogel AcA 54 was purchased from LKB. CM-Sephadex C-25 was purchased from Pharmacia and Tris, glycine, and sodium dodecyl sulfate were purchased from Bio-Rad.

Spectrapor dialysis membrane and ultrafiltration membrane were purchased from Spectrum Medical Industries INC. and Amicon Co., respectively. All other chemicals were of the highest quality obtainable.

Synthesis of de-dimethylaminotetracycline

The dimethylamino radical of the C4 position of A ring of the tetracycline molecule was chemically removed by modified method of Golub's (Golub *et al.*, 1987). The modified chemical structure was confirmed by UV and NMR spectrum comparing with the original tetracycline.

Purification of human neutrophil elastase

Human neutrophil was purified from human peripheral blood of healthy volunteer donors as described previously (Jeong *et al.*, 1987; Ghim *et al.*, 1989). Separated neutrophils were homogenized and centrifuged at 300 g. The supernatant was then chromatographed by two steps with Ultrogel AcA 54 and CM-sephadex C-25. Final product was confirmed by SDS-PAGE.

Elastase assay for inhibition study

Inhibition experiments were carried out by mixing 10 μl (=3.71 μg, specific activity was 5.06×10^2 $\mu M \cdot mg^{-1} \cdot min^{-1}$) of purified elastase, with varing amount of inhibitors in 200~300 μl of reaction medium containing 50 mM Tris-Cl, 150 mM NaCl, and 5 mM $CaCl_2$, pH 7.3. The mixtures were preincubated for 1 min at 37℃, then the substrate, SANA, was added to the reaction mixtures and incubated for proper period. The activity of elastase was measured spectrophotometrically at 410 nm by monitoring concentration of liberated p-nitroaniline, using a Titertek Multiskan Spectrophotometer (MCC/340, Flow laboratories, Switzerland). Percent inhibition was determined by $100\times[1-(V_{\text{inhibitor present}}/V_{\text{inhibitor absent orcontrol}})]$.

To calculate kinetic parameters, Lineweaver-Burk plot was used (Segel, 1974; Segel, 1975).

RESULTS

Inhibition effects of tetracyclines on the activity of elastase showed in Fig. 1. Structural different antibiotics; tetracycline, oxytetracycline, and demeclocycline inhibited HNE effectively. Demeclocycline inhibited about 18% of control activity of HNE at the concentration of less than 0.6 mM. At the same concentration, oxytetracylcine and tetracycline inhibited 42.1% and 31% of HNE, respectively.

The structures of tetracycline (Fig. 2), tetracycline-methiodide, the intermediator (Fig. 3), and de-dimethylaminotetracycline (Fig. 4) were identified by UV or/and NMR spectrum. Fig. 5

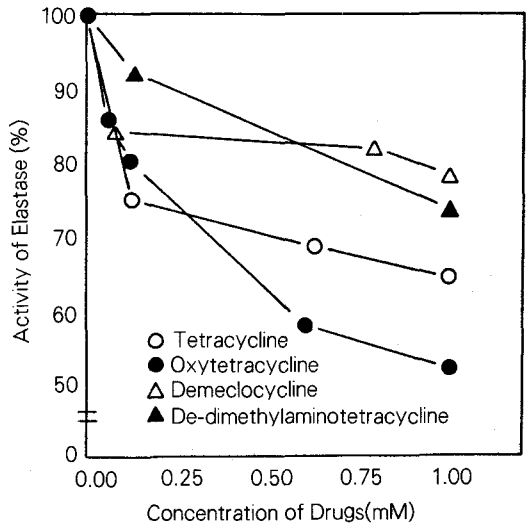

Fig. 1. Effects of tetracyclines on activity of human neutrophil elastase.

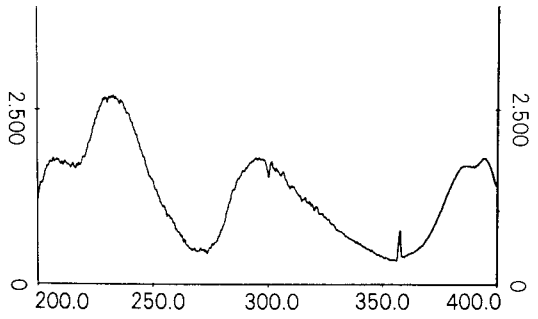

Fig. 2. Ultraviolet absorption spectra of tetracycline in 0.1N H_2SO_4 (aq.).

showed the NMR spectrum of de-dimethylaminotetracycline that dimethylamino radical of tetracycline was removed. The modified de-dimethylaminotetracycline showed no antibiotic effects on E. coli, S. aureus, and U. urealyticum (Bae *et al.*, 1991).

Fig. 6 showed inhibitory effects of de-dimethylaminotetracycline, tetracycline-methiodide and tetracycline on the activity of HNE. Both of the tetracycline and de-dimethylaminotetracycline inhibited the activity of HNE effectively, however tetracycline-methiodide showed no effect. Especially, de-dimethylaminotetracycline,

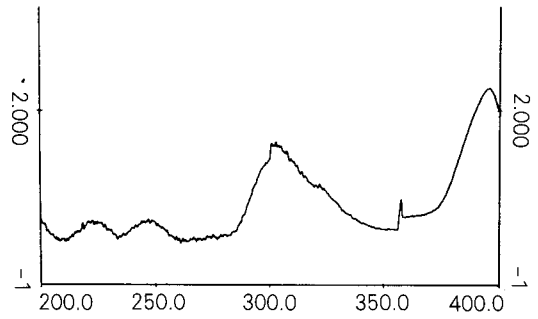

Fig. 3. Ultraviolet absorption spectra of tetracycline-methiodide in 0.1N H_2SO_4 (aq.).

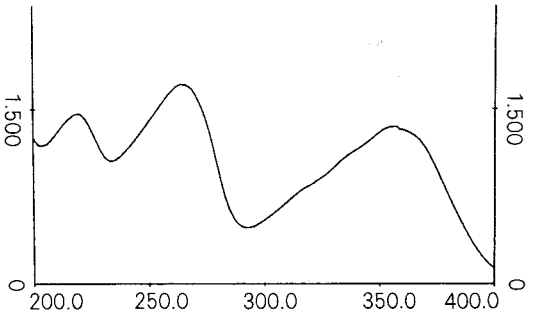

Fig. 4. Ultraviolet absorption spectra of de-dimethylaminotetracycline in 0.1N H_2SO_4 (aq.).

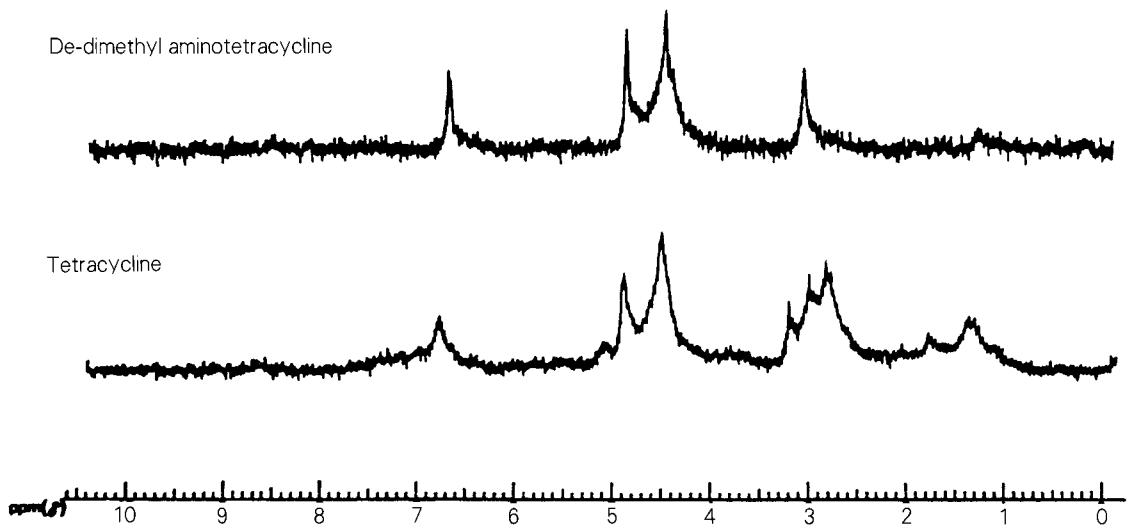

Fig. 5. NMR Spectrum of tetracycline, de-dimethylaminotetracycline in D_2O.
Spectrum amp.: 6.5 × 1000, sweep width: 10 ppm, sweep time: 5 min

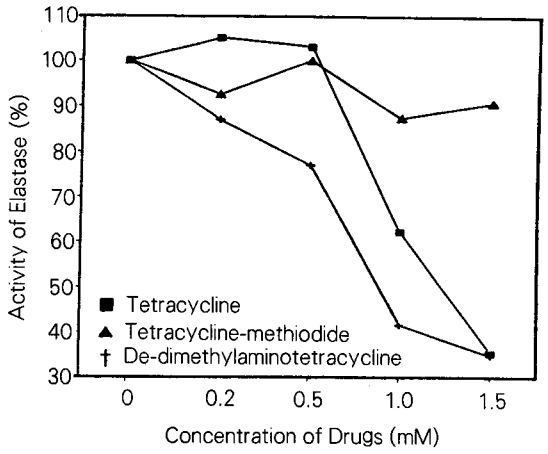

Fig. 6. Effects tetracycline-methiodide, de-dimethylaminotetracycline and tetracycline on activity of human neutrophil elastase.

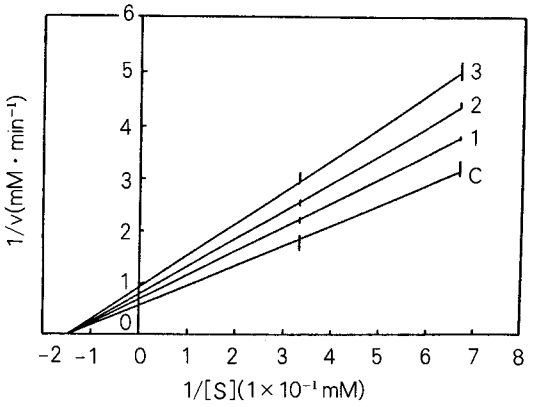

Fig. 8. Lineweaver-Burk plot of inhibition of human neutrophil elastase by Tetracycline.
Tetracycline concentrations (mM): c=control, 1=1.25, 2=2.5, 3=6.25

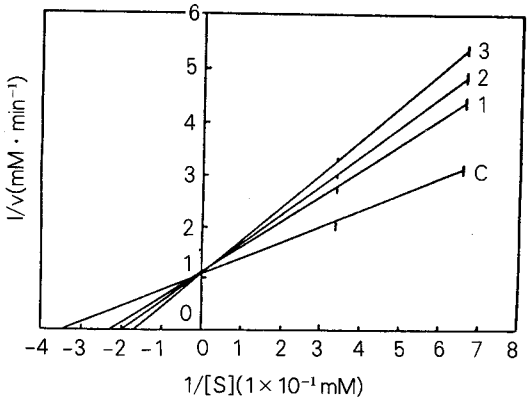

Fig. 7. Lineweaver-Burk plot of inhibition of human neutrophil elastase by oxytetracycline.
Oxytetracycline concentrations (mM): c=control, 1=0.6, 2=1.2, 3=2.4

which was removed the active antibiotic site and showed no antibiotic effect, inhibited up to 60% of the total activity of HNE at the concentration of less than 1.0 mM.

To investigate the kinetic parameters and the type of inhibition, we used Lineweaver-Burk plot. Oxytetracycline showed competitive inhibition (Fig. 7), however, tetracycline showed noncompetitive inhibition (Fig. 8). Ki values of oxytetracycline and tetracycline were 0.39 mM and 4.9 mM, respectively.

DISCUSSION

Tetracyclines are known as a antimicrobial agent which inhibits protein synthesis of microorganisms. However, we previously reported that some antibiotics including tetracyclines showed inhibition effect on the activity of HNE. To investigate possible mechanism of inhibition of HNE, we examined inhibition effect of three different sorts of tetracyclines related to their individual chemical structures (Fig. 1). Fig. 9 showed structural differences between these drugs that carboxyl radical, hydroxyl radical and chloride radical added to or removed from the side chains of original tetracycline. Demeclocycline has the chemical structure that methyl radical of the position 6# was removed and chloride radical was added to the position 7# of side chain. The drug showed lower inhibitory effect on HNE than tetracycline did. However, oxytetracycline, which added hydroxyl radical to the position 5# of tetracycline, showed the most potent inhibition effect. From this results, we suggested that the inhibition

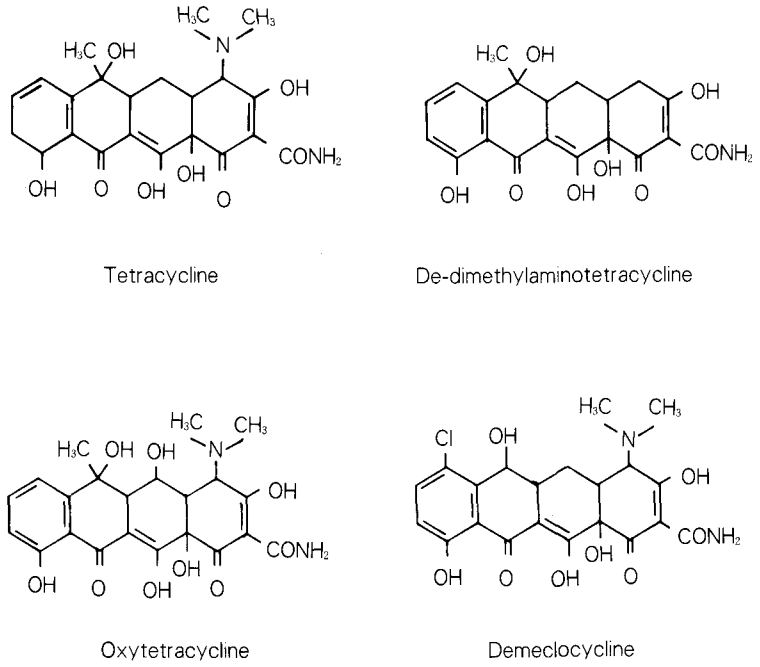

Fig. 9. Structures of tetracycline, oxytetracycline, demeclocycline and de-dimethylaminotetracycline.

effect of oxytetracycline on the activity of HNE may not be related to the main structure of the drugs rather related to the side chain, especially hydroxyl radical.

It was interesting point to investigate whether the active site for the antibiotic effect was the same site for the inhibition of the activity of the HNE or not. We synthesized de-dimethylaminotetracycline, which showed no antibiotic effect since the active dimethylamino radical was removed from the position 4# of the tetracycline. We confirmed the modified structure of synthetic product by NMR and/or UV spectrum, and compared inhibition effects of these drugs on the activity of HNE with that of original tetracycline. De-dimethylaminotetracycline showed similar inhibition effect on the activity of HNE to that of tetracycline (Fig. 6). This result strongly indicated that the active site for the antibiotic effect was not the same active site for the inhibition on the activity of the HNE.

We examined the type of inhibition by tetracycline and oxytetracycline, which showed the most potent inhibition effect. Tetracycline inhibited HNE noncompetitively, however, oxytetracycline inhibited HNE competitively. Oxytetracycline, which hydroxyl radical added to its side chain, showed the different type of inhibition on HNE. We speculated that oxytetracycline may directly influenced the hydroxyl radical of serine at the active site of HNE so as to effect to the catalytic activity of enzyme against the substrate. We suggested that the hydroxyl radical at side chain of oxytetracycline may has strong affinity with nitrogen atom of histidine which has an important role in the "charge relay system" (Blow *et al.*, 1969) and the charge relay system may not work normaly. Still we have to do fine experimental work on the details for the mechanism of action of tetracyclines on the activity of the HNE.

Overall, we speculated that inhibition of human neutrophil elastase by tetracyclines was not depended on the dimethylamino radical which is a critical active site for antibiotic effect, rather it was depended on the hydoxyl radical at the side chain of tetracyclines. Thus, we susggested that

tetracyclines, which showed inhibition effect on the activity of HNE, possibly be a part of mechanism of action which might be related to direct inhibition of elastase at the infected sites beside of their known mechanism of action against the microorganisms. The property of inhibition effect on the activity of HNE of tetracyclines might be an additional molecular mechanism of action of these drugs at the inflammatory sites. Furthermore, structural modified tetracycline, de-dimethylaminotetracycline may have some advantages to apply for the treatment of chronic inflammatory diseases which were caused by elastase without any development of resistance against microorganisms.

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=국문초록=

Tetracycline계 항균제에 의한 호중구 Elastase의 효소 활성도 억제 및 그 작용 기전

고신대학교 의과대학 약리학교실

김 우 미·강 구 일

Tetracycline계 약제가, 류마치양 관절염을 비롯한 염증성 질환들의 주된 병인으로 알려지고 있는 호중구 elastase의 활성도를 억제하였으며, 특히 oxytetracycline, demeclocycline, 그리고 tetracycline 등은 분자 구조적 차이에 따라 elastase의 효소 활성도에 대하여 다양한 억제율을 나타내었다. 측쇄 구조의 5번 위치에 OH^-기가 첨가된 oxytetracycline이 가장 높은 억제율을 나타내었다. 억제 양상에 있어서도 tetracycline이 비경쟁적 저해 형태를 보인 반면에, oxytetracycline은 경쟁적 저해 형태를 나타내었으며, Ki값은 각각 4.9 mM과 0.39 mM로 산출되었다. 또한 항균 효과를 나타내는 활성 부위를 제거시킨 de-dimethylaminotetracycline을 합성하여 효소 활성도 억제 실험에 사용한 결과, tetracycline과 유사한 효소 억제 작용을 나타냄을 확인하였다. 이상의 연구 결과에서, tetracycline의 효소 활성도 억제 작용은 항균 효과를 나타내는 활성 부위와 상관없이 독립된 기전에 의해서 일어나는 약리 작용이며, 측쇄 구조의 OH^-기가 이 작용에 영향을 주는 일부 원인인 것으로 추정할 수 있으며, 이를 tetracycline계 약제가 염증 부위에서 나타내는 분자 단계에서의 새로운 약리 기전으로 제시하고자 한다. 또한 de-dimethylaminotetracycline은 항균제의 장기 사용시에 발생할 수 있는 저항균의 출현과는 무관하므로, 다른 부작용에 대한 연구가 선행될 경우, elastase에 의해 야기되는 만성 질환들의 치료제로써 중요한 역할을 할 것으로 사료된다.