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# Effective Antibacterial Action of Tat (47–58) by Increased Uptake into Bacterial Cells in the Presence of Trypsin

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**In a previous study, we found an antifungal effect on human pathogenic fungi by the cell-penetrating peptide Tat (47–58) derived from HIV-1. Tat (47–58) immediately entered into the fungal nucleus and affected some physiological changes on the intracellular condition. In this study, Tat (47–58) showed a broad spectrum of antibacterial activity against pathogenic bacteria including bacterial clinical isolates. To improve resistance against proteases for use *in vivo*, we synthesized an analog of Tat (47–58) by substituting the L-amino acid for the D-amino acid. The D-enantiomer of Tat (47–58) also exhibited a broad spectrum of antibacterial activity at almost the same level of L-Tat (47–58) concentration. Unlike L-Tat (47–58), D-Tat (47–58) showed a significant proteolytic resistance against all proteases tested and antimicrobial activities in the presence of trypsin. Moreover, D-Tat (47–58) inhibited MRSA infection in human HeLa cells whereas L-Tat (47–58) partially allowed MRSA infection, and the results were due to the proteolytic resistance of D-Tat (47–58).**

**Keywords:** Tat (47–58), cell-penetrating peptide, antibacterial activity, proteolytic resistance, MRSA infection

In treating bacterial infections, the existence of several clinical isolates has become a significant problem because of their drug resistance to conventional antimicrobial agents, and the emergence of drug-resistant strains has accelerated the development of novel antimicrobial agents with novel acting mechanisms. Cationic antimicrobial peptides (AMPs) are small molecules with a broad spectrum of antimicrobial activity against microbial pathogens, and they play a key protective role in the innate immunity of host organisms [13, 28]. Most AMPs display rapid-killing activity toward bacterial cells displaying *in vitro* membranelytic actions [1, 10], such as melittin [32], ceratotoxin [2], pleurocidin [22], and cecropin [7].

Recently, the cell-penetrating peptides (CPPs) have been extensively studied regarding their material delivery system in various mammalian cells [16], and several studies on the actions of CPPs in microorganisms have been reported. CPPs target intracellular molecules, such as DNA, RNA, and proteins, after translocating the bacterial membranes. Indolicidin, which consists of 13 residues from cytoplasmic granules of bovine neutrophils, inhibits DNA synthesis [25], and PR-39, which is isolated from the small intestine of the pig, inhibits DNA and protein syntheses [4]. CPPs have potential as novel antimicrobial agents with a novel antibiotic mechanism, which is distinct from the membrane-disturbing action of most AMPs.

Tat (47–58) is an Arg-rich peptide (YGRKKRRQRRRD) derived from the Tat protein of HIV-1, which activates viral replication [21]. In a previous study, we reported the antifungal action of Tat (47–58) peptide toward several fungal pathogens [14]. Tat (47–58) penetrated the fungal cell membrane in a time-, temperature-, and salt-independent manner. The localization of Tat (47–58) in the nucleus leads to the cell cycle arrest and cell death in fungal cells.

In this study, we will discuss the antibiotic actions of Tat (47–58) on human pathogenic bacteria, including antibiotic-resistant bacterial strains clinically isolated, and the *in vitro* activity for the inhibition of bacteria infection in mammalian cells. However, Tat (47–58) consists of several arginine residues, which are sensitive to proteases in serum. This property is significantly lethal to antimicrobial peptidic agents, which need its stable action after clinical treatment. To overcome this protease-sensitive property, we will also discuss the improvement of proteolytic resistance to several proteases in human beings.

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## MATERIALS AND METHODS

### Peptide Synthesis

The peptides were synthesized by a solid-phase method using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry [31]. The coupling of Fmoc amino acids was performed with *N*-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC). Amino acid side chains were protected as follows: *tert*-butyl (Asp), trityl (Gln), and *tert*-butyloxycarbonyl (Lys). Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethanedithiol, and triisopropylsilane (88:2.5:2.5:2.5:2.5:2.0, v/v) for 2 h at room temperature. The crude peptide was repeatedly washed with diethylether, dried in a vacuum, and purified using a reversed-phase preparative HPLC on a Waters 15-μm Deltapak $C_{18}$ column (19×30 cm). The purity of the peptide was checked by an analytical reversed-phase HPLC on an Ultrasphere $C_{18}$ column (Beckman, U.S.A.), 4.6×25 cm. The purified peptides were hydrolyzed with 6 N HCl at 110°C for 22 h, and then dried in a vacuum. The residues were dissolved in 0.02 N HCl and subjected to an amino acid analyzer (Hitachi Model 8500 A, Japan) for peptide concentration. The molecular weights of the synthetic peptides were determined using matrix-assisted laser desorption ionization (MALDI)-mass spectrometry.

### Bacterial Strains and Growth Conditions

*Bacillus subtilis* (KCTC 1918), *Staphylococcus epidermidis* (KCTC 1917), *Staphylococcus aureus* (KCTC 1621), *Escherichia coli* (KCTC 1682), *Proteus vulgaris* (KCTC 2433), *Pseudomonas aeruginosa* (KCTC 1637), and *Salmonella typhimurium* (KCTC 1926) were grown in Mueller-Hinton (MH) broth (Difco, MD, U.S.A.) at 37°C with shaking at 140 rpm. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains and multidrug-resistant *Pseudomonas aeruginosa* (MRPA) strains were obtained from Kyungpook National University Hospital, and were grown in MH broth at 37°C with shaking at 140 rpm.

### Antibacterial Susceptibility Test

An antibacterial susceptibility test was determined according to the NCCLS guideline [17], and the minimum inhibitory concentration (MIC) was defined as the lowest concentration of visible growth after an overnight incubation in MH broth at 37°C. The inoculum size was $10^6$ CFU/ml.

### Hemolytic Activity Assay

The hemolytic activity of the peptides was evaluated by measuring the released hemoglobin of 4% suspension of human red blood cells (RBCs) at 414 nm [3, 29]. Human RBCs were washed with PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.0) three times. Human RBCs (100 μl) diluted in PBS to 8% (v/v) were seeded on 96-well plates, and then 100 μl of the two-fold diluted peptide solution (from 20 to 1.25 μM) was added to each well. After incubation for 1 h at 37°C, aliquots (100 μl) were transferred to a new 96-well plate, and the hemoglobin release was measured by the absorbance at 414 nm with an ELISA plate reader (Molecular Devices Emax, California, U.S.A.). PBS and 0.1% Triton X-100 were used as agents to establish 0 and 100% hemolysis, respectively. The hemolysis percentage was calculated by employing the following equation:

$$\text{Percentage hemolysis} = [(\text{Abs}_{414\,nm} \text{ in the peptide solution} - \text{Abs}_{414\,nm} \text{ in PBS}) / (\text{Abs}_{414\,nm} \text{ in 0.1\% Triton X-100} - \text{Abs}_{414\,nm} \text{ in PBS})] \times 100.$$

### Proteolytic Resistance of Enantiomeric Peptides

A solution containing either a mixture of trypsin (E.C. 3.4.21.4., Sigma; 5 μg), plasmin (E.C. 3.4.21.7., Sigma; 5 μg), carboxypeptidase B (E.C. 3.4.17.2., Sigma; 5 μg), or V8 protease (E.C. 3.4.21.19., Sigma; 5 μg) was reacted with each enantiomer (1 μg each) in 10 mM sodium phosphate buffer (pH 7.6), and a solution containing pepsin (E.C. 3.4.23.1., Sigma; 5 μg) was reacted with the peptides in 20 mM sodium acetate buffer (pH 4.0). After incubation for 2 h at 37°C, the peptides were separated on 16.5% tricine-SDS-PAGE and detected by Coomassie blue staining.

The peptide solution containing MIC of each peptide was incubated with trypsin (5 μg) for 2 h at 37°C, and the protease inhibitor aprotinin (Sigma; 4 μg) was added and incubated for 1 h at 37°C. The peptide solutions were suspended in the cultures of bacterial cells and were incubated at 37°C overnight. The cultures were streaked on 1.5% MH agar plates, divided into three equal parts, and incubated at 37°C overnight.

### Fluorescence Microscopy

To obtain FITC-labeled peptide, fluorescein isothiocyanate (FITC) was added to a 0.5 mg/ml peptide solution in 100 mM sodium bicarbonate (pH 9.3), and the FITC-labeled peptides were purified by a gel-filteration column chromatography using a 12 cm Sephadex G-50 column. The cellular localization of FITC-labled Tat (47–58) in bacterial cells was analyzed by a confocal laser scanning microscopy. The cells were incubated in MH medium for 18 h at 37°C, and washed with an ice-cold PBS buffer. After the bacterial cells were washed, the cells were treated with 1 μM of the FITC-labeled peptides and incubated for 1 h at 37°C. Visualization and localization of the labeled peptides were performed by a Laser Scanning Spectral Confocal Microscope (Leica TCS SP2; Leica, Swiss) [20, 23].

### FACScan Analysis

For analysis of the intracellular integrity after peptide treatment, MRSA cells ($2\times10^9$ cells in MH media) were first harvested at the log phase. Each 20 μM of FITC-labeled peptide was pre-incubated with or without trypsin for 1 h at 37°C and was added to the MRSA cultures. The suspensions were incubated for a further 1 h at 37°C under constant shaking (140 rpm). After incubation, extracellular FITC-labeled peptides were removed through excessive washing with ice-cold PBS. The fluorescence of FITC was monitored in the FL2-H channel. FACScan analysis was performed by the FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.).

### HeLa Cell Culture, MRSA Infection, and Enantiomeric Tat (47–58) Treatment

HeLa cells were cultured in a minimal essential medium α (MEM α) supplemented with 10% fetal calf serum (FCS). Before MRSA infection, the HeLa cells were suspended by scraping, to avoid trypsin, transferred to 6-well plates, and grown to ~30% confluence. Freshly grown MRSA cells were added at $10^7$ cells/ml. After MRSA infection, 50 μM of the peptides were added immediately and the plates were incubated for 24 h at 37°C in 5% $CO_2$.

## RESULTS

### Antibacterial Effect and Hemolytic Effect on Human Erythrocytes

Recently, some CPPs have shown significant lethal effects on microbial species, and these properties of the peptides make them a good candidate for antimicrobial agents [9, 8]. To investigate the antibacterial activity of Tat (47–58) on bacterial pathogens, we examined antibacterial activity in several bacterial strains by the NCCLS method [17]. Tat (47–58) exerted toxic activity toward all bacterial strains including antibiotic-resistant isolates, at 5–20 μM of peptide concentration, except for MRSA 3 (Table 1). The MIC values of Tat (47–58) indicated a broad spectrum of antibacterial activity in microbial pathogens.

Many antimicrobial peptides have been discovered all over the world, but they are not yet absolute candidates for a therapeutic agent because of human toxicity, especially membrane-lytic action. To assess the toxicity [11, 15], the hemolysis of human RBCs by enantiomeric Tat (47–58) was determined by evaluating the hemoglobin release at 414 nm. Tat (47–58), in the range of 1.25–20 μM, did not show the hemolysis of RBCs (Table 2).

### Proteolytic Resistance of D-Tat (47–58)

The main shortcoming of the peptidic agents emerging in the development of a therapeutic agent is sensitivity to the proteases in a human body. Tat (47–58) has six Arg and two Lys, which are sensitive amino acids to several proteases, such as trypsin. To enhance the resistance of Tat (47–58) against proteases, we synthesized an enantiomeric peptide consisted of D-amino acids and tested the antibacterial activity. D-Tat (47–58) showed no differences in its MIC values compared with those of L-Tat (47–58) (Table 1).

**Table 1.** Antibacterial activity of L- and D-Tat (47–58).

| Bacterial strains | MIC[a] (μM) | |
|---|---|---|
| | Peptides | |
| | L-Tat (47–58) | D-Tat (47–58) |
| *B. subtilis* | 20 | 20 |
| *S. epidermidis* | 10 | 5 |
| *S. aureus* | 10 | 10 |
| *E. coli* | 10 | 10 |
| *P. vulgaris* | 5 | 5 |
| *P. aeruginosa* | 10 | 10 |
| *S. typhimurium* | 5 | 5 |
| MRSA[b] 1 | 20 | 20 |
| MRSA 2 | 10 | 10 |
| MRSA 3 | 0.625 | 0.313 |
| MRPA[c] 1 | 20 | 10 |
| MRPA 2 | 20 | 20 |
| MRPA 3 | 20 | 10 |

[a]MIC (minimum inhibitory concentration) was determined by the microdilution method of the NCCLS guideline.
[b]MRSA (methicillin-resistant *Staphylococcus aureus*); we used a clinical isolate.
[c]MRPA (multidrug-resistant *Pseudomonas aeruginosa*); we use a clinical isolate.

**Table 2.** Hemolytic activity of L- and D-Tat (47–58) against human RBCs.

| Peptides | % Hemolysis[a] (μM) | | | | |
|---|---|---|---|---|---|
| | 20 | 10 | 5 | 2.5 | 1.25 |
| L-Tat (47–58) | 0 | 0 | 0 | 0 | 0 |
| D-Tat (47–58) | 0 | 0 | 0 | 0 | 0 |
| Melittin | 100 | 100 | 95.4 | 61.1 | 34.5 |

[a]% Hemolysis=[($Abs_{414\,nm}$ in the peptide solution − $Abs_{414\,nm}$ in PBS)/($Abs_{414\,nm}$ in 0.1% TritonX-100 − $Abs_{414\,nm}$ in PBS)]×100.

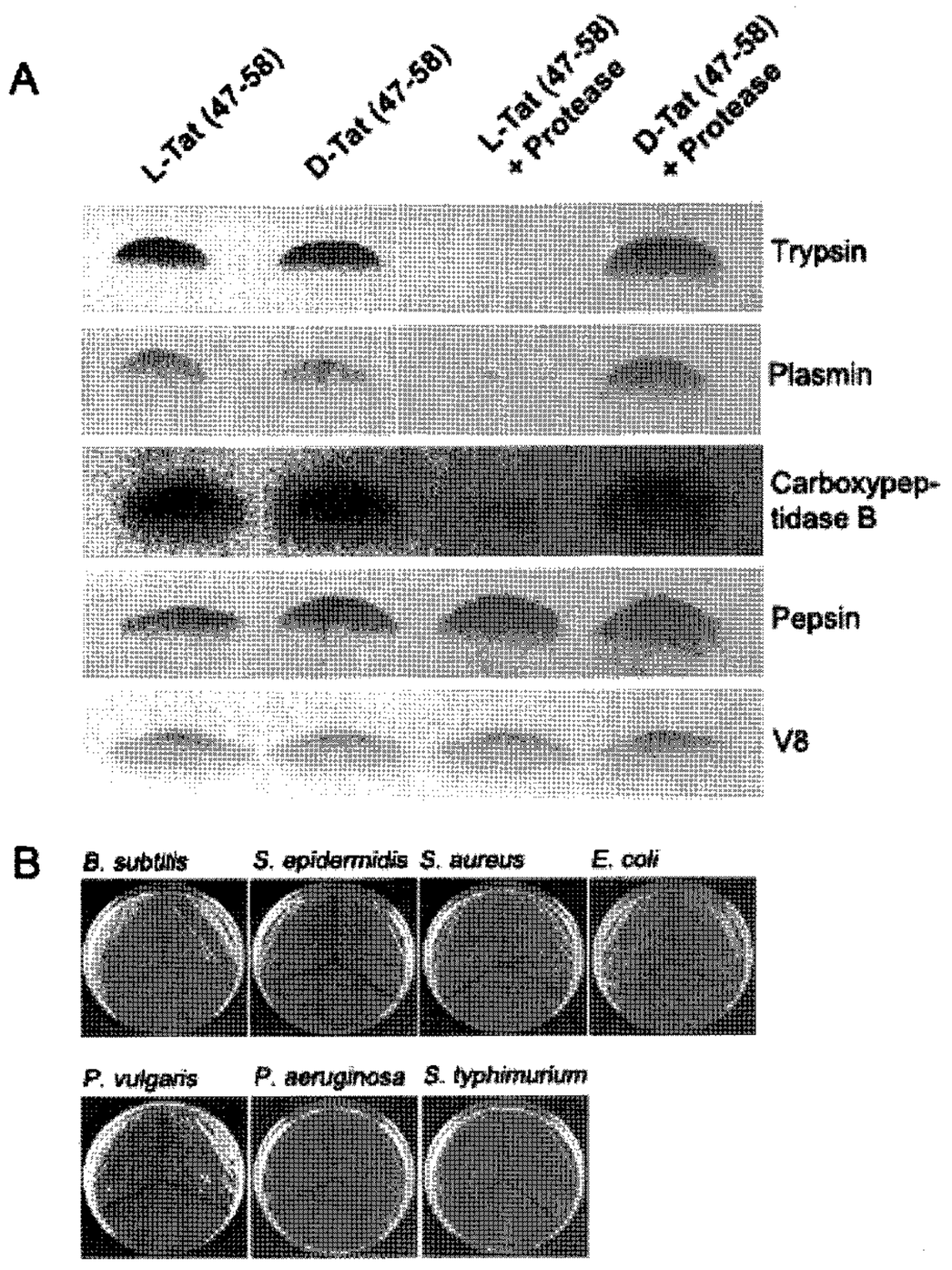


**Fig. 1.** Effect of proteases on enantiomeric Tat (47–58) by 16.5% tricine-SDS-PAGE, and minimum inhibitory concentration (MIC) of L- and D-Tat (47–58) against bacterial cells in the presence of trypsin.
Each enantiomeric Tat (47–58) was incubated in the presence or absence of the tested protease for 2 h at 37°C, and then 16.5% tricine-SDS-PAGE was performed (**A**). Each peptide was incubated with trypsin for 2 h at 37°C, and then the activity of trypsin was inhibited by a protease inhibitor, aprotinin, through incubation for 1 h at 37°C. These suspensions were added to bacterial cultures and incubated at 37°C overnight, and then the cultures were incubated on the 1.5% MH agar plate, divided into three sectors (**B**): no peptide treated (upper left sector), L-Tat (47–58) treated (upper right sector), and D-Tat (47-58) treated (lower sector).

Moreover, D-Tat (47–58) exhibited no hemolytic activity against human RBCs in the tested concentration range (Table 2). The result indicated that the transition from L-Tat (47–58) to D-Tat (47–58) does not make it possible to obtain toxicity on human erythrocytes. We examined the proteolytic resistance of two enantiomeric peptides by tricine-SDS-PAGE, and the effect of the proteases on the antibacterial activity of the peptides. As shown in Fig. 1A, some proteases targeting specific amino acids, such as the C-terminus of Arg and Lys, remarkably degraded L-Tat (47–58). L-Tat (47–58) was also resistant to other proteases specific to amino acids, which do not consist of Tat (47–58). Whereas L-Tat (47–58) was sensitive to trypsin, plasmin, and carboxypeptidase B, D-Tat (47–58) showed significant resistance to all proteases tested.

The proteolytic resistance of the peptides resulted in the difference of activity in bacterial strains (Fig. 1B). After the pre-incubation of peptide and trypsin for 2 h at 37°C, trypsin was inhibited by aprotinin and a bacterial culture was added to these suspensions. Trypsin significantly inactivated L-Tat (47–58) by degrading the peptide. However, D-Tat (47–58) was resistant to trypsin and maintained its lethal activity in bacterial cells. These results indicated that Tat (47–58) obtained its proteolytic resistance by the substitution of D-amino acids, and the enantiomeric conversion did not affect the activity of Tat (47–58).

## Intracellular Distribution of Tat (47–58) Within Bacterial Cells and the Effect of Protease on the Uptake of the Peptides

To investigate the uptake of the two enantiomeric peptides into bacteria, we examined for fluorescence localization in the bacterial cells using fluorescence microscopy. Each MRSA and *E. coli* cells were incubated with FITC-labeled

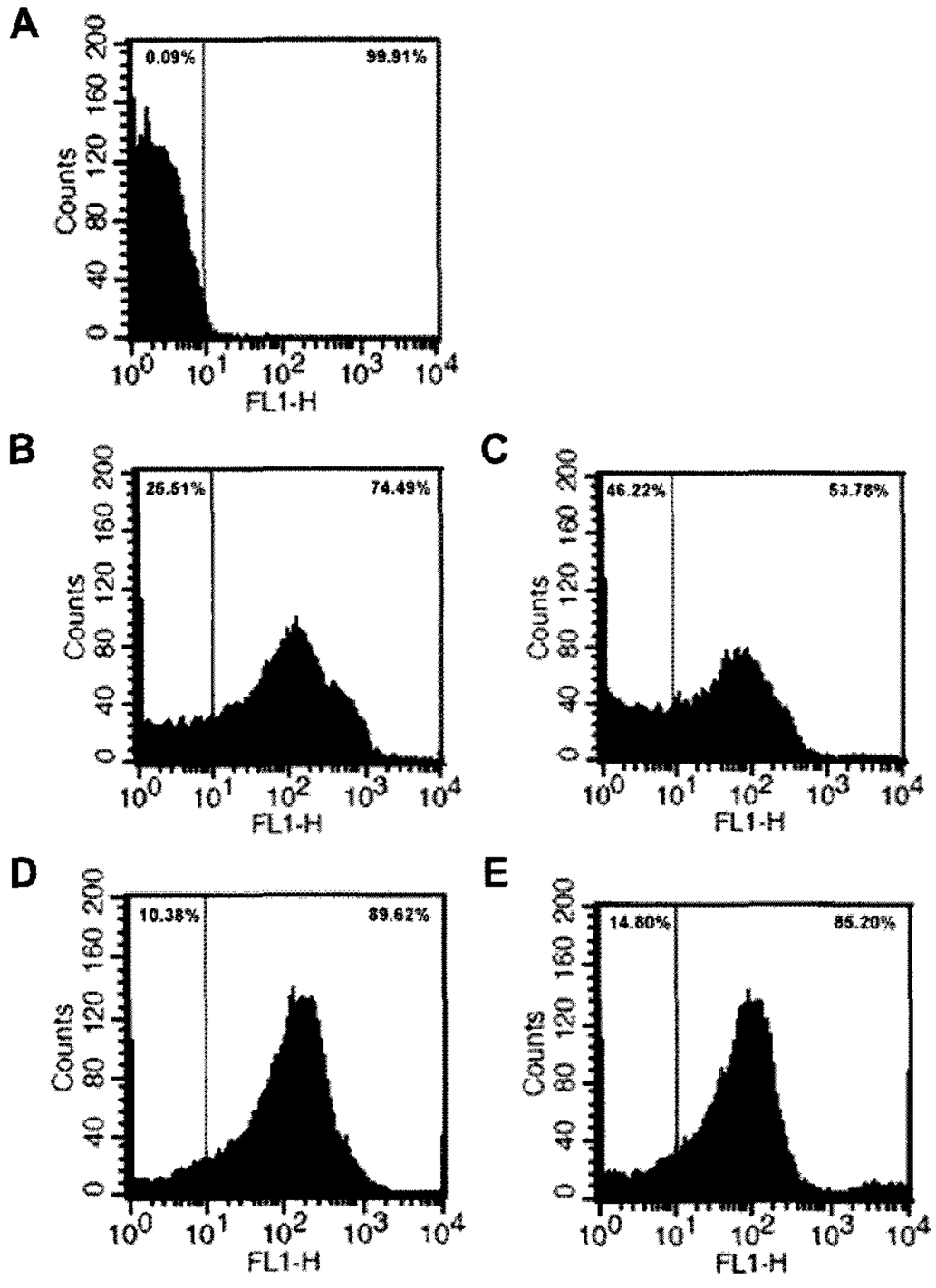


**Fig. 3.** Entry efficiency of two enantiomeric Tat (47–58) pre-incubated with trypsin investigated by FACScan analysis.
Each FITC-labeled peptide was incubated with trypsin for 1 h at 37°C and was added to the MRSA cultures. After incubation for 1 h at 37°C, FACScan analysis was performed. **A**. Naive MRSA cells; **B**. L-Tat (47–58)-treated MRSA cells without pre-incubating with trypsin; **C**. L-Tat (47–58)-treated MRSA cells with pre-incubating with trypsin; **D**. D-Tat (47–58)-treated MRSA cells without pre-incubating with trypsin; **E**. D-Tat (47–58)-treated MRSA cells with pre-incubating with trypsin.

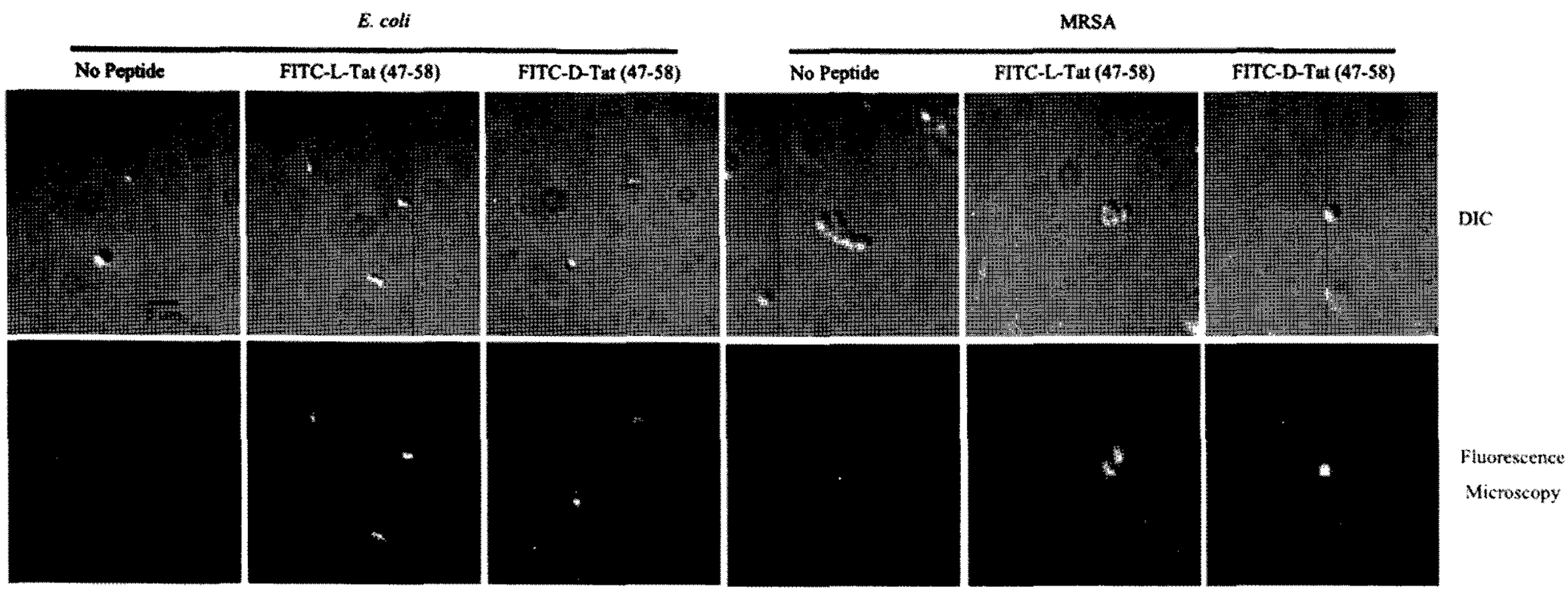


**Fig. 2.** Accumulation of FITC-labed peptides by fluorescence microscopy.
The bacterial cells, cultured for 18 h at 37°C and washed with PBS, were treated with 1 μM of FITC-labeled peptides and incubated for 1 h at 37°C. After incubation of the suspension, the cells were harvested and washed with PBS several times.

peptides for 1 h at 37°C, and the cells were washed with PBS. As shown in Fig. 2, remarkable intracellular fluorescence was observed in the tested bacteria, Gram-positive bacteria MRSA 1 and Gram-negative bacteria *E. coli*.

We also examined the capacity of the peptides in the presence of trypsin, which induced the decrease of antibacterial activity by the degradation of L-Tat (47–58) (Fig. 3). After pre-incubation of the FITC-labeled peptides and trypsin, the fluorescence intensity from the MRSA cells treated with fluorescence-labeled peptide was detected through FACScan analysis. In the absence of trypsin, L- and D-Tat (47–58) had accumulated in the cells, about 74% (Fig. 3B) and 89% (Fig. 3D), respectively. As in the previous experiment, D-Tat (47–58) nearly maintained the capacity to enter the cells, showing its intensity at about 85% (Fig. 3E). However, accumulated L-Tat (47–58) in the cells was decreased significantly by the proteolytic activity of trypsin, which exhibited the decrease of fluorescence intensity from about 74% to about 53% (Fig. 3C). Although the 50% intensity of L-Tat (47–58) in the presence of trypsin could not explain the intact loss of activity, it seems to be caused by FITC-labeled fragments degraded by trypsin. Nevertheless, the difference of intensity between L-Tat (47–58) and D-Tat (47–58), pre-incubated with trypsin, was over 30%. Therefore, these results indicated that the protease sensitivity of L-Tat (47–58) decreased antibacterial activity in the presence of proteases, owing to the reduction of entry into the cells by a degradation of peptides.

### Inhibition of MRSA Infection in HeLa Cells

Exerting selective antimicrobial activity is an important matter for peptidic antibiotic agents in therapy [12]. To investigate the antibacterial activity of enantiomeric Tat (47–58) for protecting against bacterial infection in mammalian cells, MRSA-infected HeLa cells were observed after incubation in the presence of each enantiomer of Tat (47–58) for 24 h at 37°C (Fig. 4). We inoculated $10^7$ cells/ml of MRSA to a HeLa cell culture, and immediately treated with each enantiomer of Tat (47–58) at 50 μM. D-Tat (47–58) significantly protected against the MRSA infection in the HeLa cell, after 24 h from the infection (Fig. 4D). However, L-Tat (47–58) did not perfectly prevent the infection, considering the rounded HeLa cells were induced by the MRSA infection (Fig. 4B). The two peptides exhibited the prevention of the MRSA infection without the effects on the HeLa cell growth at 50 μM. Thus, the results demonstrated that Tat (47–58) exerted antibacterial activity against MRSA in mammalian cell, without the cytotoxicity of the peptide concentration affecting mammalian cell growth, and D-formed Tat (47–58) was more active in preventing bacterial infection, based on the increased stability due to proteolytic resistance.

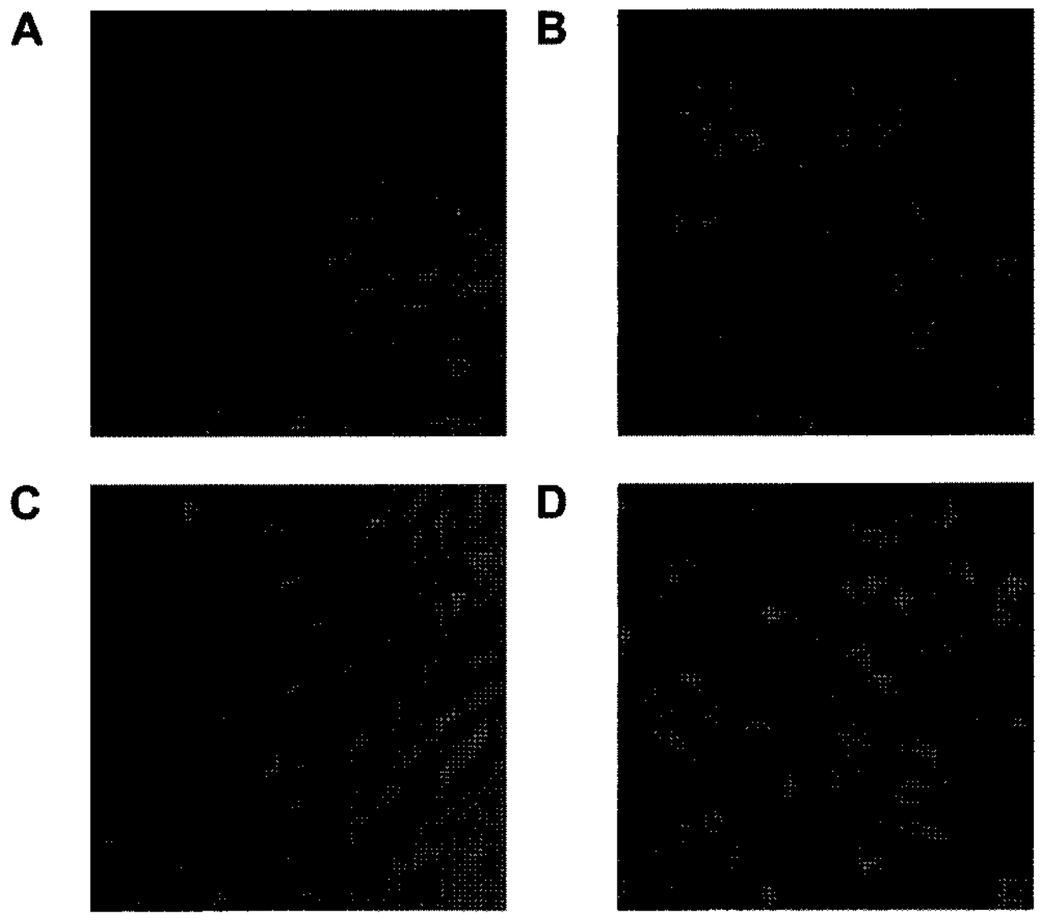


**Fig. 4.** MRSA infection of HeLa cells in the presence and absence of enantiomers of Tat (47–58).
HeLa cells were cultivated in MEM α supplemented with 10% FCS. After preparation of HeLa cells, the HeLa cells were infected with MRSA cells. After infection, 50 μM of the peptides was added immediately and the plates were incubated for 24 h at 37°C with continuously circulating 5% $CO_2$. **A**. Non-infected HeLa cells in the presence of L-Tat (47–58); **B**. MRSA-infected HeLa cells in the presence of L-Tat (47–58); **C**. Non-infected HeLa cells in the presence of D-Tat (47–58); **D**. MRSA-infected HeLa cells in the presence of D-Tat (47–58).

## Discussion

Antimicrobial CPPs have been studied to reveal their mechanism in the penetrating process and their effect on the growth of microorganisms [18]. Some CPPs have shown a significant antimicrobial effect on pathogenic microorganisms [4], and various effects on intracellular molecules inhibiting their growth of them [25, 28, 30]. Tat (47–58) is a cell-penetrating domain derived from HIV-1 Tat, which is a trans-activator for the transcription of the HIV-1 gene in the intracellular environment of hosts [25, 16]. We had reported that Tat (47–58) showed an antifungal effect on fungal pathogens in humans, and its acting mode in the process leads to the fungal cell death [14]. Owing to the antifungal effect of Tat (47–58), we examined its antibacterial effect on several bacterial pathogens including drug-resistant isolates, and also investigated the proteolytic resistance of its enantiomeric peptide by substitution for D-amino acid as one of the various ways that can be used to improve its stability under the environment. Sometimes, substituting L-amino acids for D-amino acids partially or wholly in natural peptide sequences makes its activity worsen or strengthen [6, 26]. The two enantiomeric Tat (47–58) peptides showed a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria, including clinical isolates, without remarkable differences

between the two enantiomeric peptides (Table 1). The result also indicated that the transition to D-enantiomer did not contribute to exert their activity. In addition, the transition did not induce hemoglobin releases of human RBCs by RBC-disturbing action of the peptides. Through the substitution for enantiomeric amino acids, it is thought that the activity of Tat (47–58) may depend on its sequence, not on its secondary structure, which plays a crucial role in the action of most antimicrobial peptides [33].

Although peptidic agents have been considered as effective therapeutic agents in the treatment for several human diseases, they have a lethal fault that weakens them to the enzymatic action of the proteases in the human body. To overcome this problem, many organisms often utilize D-amino acids in the specific part of their proteins, which is a very important component in protecting themselves from external environments [5]. D-Tat (47–58) showed a noticeable proteolytic resistance to several proteases tested with antibacterial activity (Fig. 1).

We have previously reported the intracellular localization of Tat (47–58) in *C. albicans* cells [14], and the result indicated the penetrating capacity in microorganisms. Two enantiomeric Tat (47–58) peptides labeled with fluorescence also localized in MRSA and *E. coli* cells, and accumulated remarkably. However, the permeable efficiency of L-Tat (47–58) treated with trypsin was decreased. The difference in the permeable efficiency may have been related to the loss of L-Tat (47–58) activity in the presence of trypsin. A recent article reported that the stability of cell-penetrating peptides in the extracellular environment is a critical determinant for delivery efficiency into the cell [19], and the study supports that the proteolytic resistance of the two enantiomers of Tat (47–58) causes the differences of permeable efficiency between them. D-Tat (47–58) exhibited a broad spectrum of antibacterial activity as did the natural Tat (47–58), and maintained its activity in the presence of trypsin whereas L-Tat (47–58) lost its activity. These results indicated that the stability of peptides could result in more effective activity *in vivo*, and it could contribute by protecting against bacterial infection in HeLa cells (Fig. 4). There are two important points that should be understood in order to improve Tat (47–58) as an antimicrobial agent; its entry mechanism into the microbes and how it shows specific activity in microbes is not clear. Nevertheless, an enantiomeric peptide of natural Tat (47–58) may be expected to be studied widely as a therapeutic agent in treating microbial infectious diseases, and may be a good candidate for the design of novel peptidic antibiotics with novel mechanisms.

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