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Biological activity of Tat (47–58) peptide on human pathogenic fungi

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Abstract

Tat (47–58) peptide, a positively charged Arginine-rich peptide derived from HIV-1 regulatory protein Tat, is known for a peptidic delivery factor as a cell-penetrating peptide on mammalian cells. In this study, antifungal effect and its mode of action of Tat peptide were investigated on fungal cells. The results indicate that Tat peptide exhibits antifungal activity against pathogenic fungal cells without hemolytic effect on human erythrocytes. To understand the mechanism(s) of Tat peptide, the cellular distribution of the peptide was investigated. Tat peptide internalized in the fungal cells without any damage to cell membrane when examined using an artificial liposome (PC/cholesterol; 10:1, w/w). Moreover, flow cytometry analysis exhibited the uptake of Tat peptide by energy- and salt-independent pathway, and confocal scanning microscopy displayed that this peptide accumulated in the nucleus of fungal cells rapidly without any impediment by time or temperature, which generally influence on the viral infections. After penetration into the nuclear, the peptide affected the process of cell cycle of *Candida albicans* through the arrest at G1 phase.
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Cationic antimicrobial peptides occurring naturally from many different organisms play important roles in innate immunity against various infectious pathogenic microorganisms [1]. These peptides are efficacious antimicrobials in nature with several properties, such as broad antimicrobial spectra, highly selective activity, as well as difficult resistance development which make these compounds promising candidates as novel therapeutic agents [2]. Accordingly, many of these peptides have been extensively studied to understand their antimicrobial mechanisms against pathogenic microorganisms. In many studies on the mechanism of antimicrobial peptides, it has been shown that most peptides give the lethal effect on bacterial membrane [3] and others damage critical intracellular target after the intracellular distribution of the peptides [4].

Recent reports suggested that cell-penetrating cationic peptides target some intracellular molecules for their antimicrobial effect on microorganisms. Buforin II consisting of 21 amino acids derived from buforin I inhibits the cellular functions by binding to nucleic acid [5], cecropin PR-39 isolated from pig intestine inhibits DNA and protein synthesis [6], and indolicidin isolated from cytoplasmic granules of bovine neutrophils also inhibits the synthesis of intracellular macromolecules including DNA synthesis [7]. These peptides show their antimicrobial action by a different mode of action than the well-known lytic peptides.

Tat peptide is Arg-rich cationic peptide derived from membrane-translocating basic domain of HIV-1 Tat protein, which is a potent transactivator for viral replication [8]. Various short Tat peptides have been reported to penetrate the membranes and to localize in the nucleus of HeLa cells, and the internalization is not influenced by

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low temperature, endocytosis inhibitors, and metabolic inhibitors [9,10] but the mechanism of internalization of the peptides is not clear. In addition, it suggests that the effects of temperature and metabolic inhibitors and the time and concentration dependence of cellular uptake appear to depend on the peptide sequences and cell lines [11]. Despite the great potential of Tat peptides as novel antimicrobial peptide model with other cell-penetrating antimicrobial peptides, it has not been reported about the biological action of Tat peptides, including antimicrobial action.

In this study, we report the antifungal mechanism of Tat (47–58) peptide on human pathogenic fungi and suggest a potential for providing a template for design of potent antifungal peptide possessing a novel antifungal mechanism.

Materials and methods

Peptide synthesis. The peptides were synthesized by solid-phase method using Fmoc-chemistry [12]. The crude peptides were purified by a reversed-phase preparative HPLC on a C_{18} column (20 × 250 nm, Shim-pack) using a linear gradient of 20–80% (v/v) acetonitrile in 0.1% trifluoroacetic acid for 30 min [13]. The purity of the peptides was checked by an analytical reversed-phase HPLC using a C_{18} column (4.6 × 250 nm, Shim-pack. The molecular weights of the synthetic peptides were determined using the matrix-assisted laser desorption ionization (MALDI) mass spectrometer.

Fungal strains and antifungal activity assay. Malassezia furfur (KCTC 7744), *Saccharomyces cerevisiae* (KCTC 7296), and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC), at the Korea Research Institute of Bioscience Biotechnology (KRIBB), Taejon, Korea. *Candida albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan. Antifungal activity of the peptides was determined by the broth microdilution assay [14]. The inhibition of fungal growth was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay at 580 nm with a Microplate ELISA reader (Molecular Devices Emax, California, USA) [15].

Hemolytic activity assay. The hemolytic activity of the peptides was evaluated by determining the release of hemoglobin from a 4% suspension of fresh human erythrocytes at 414 nm with an ELISA plate reader (Molecular Devices Emax, California, USA) [16]. The hemolysis percentage was calculated using the following equation: % hemolysis = $[(Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100 − $Abs_{414\ nm}$ in PBS)] × 100.

Preparation of FITC-labeled Tat peptide. Fluorescein isothiocyanate (FITC) was freshly dissolved in dimethyl sulfoxide to 10 mg/ml and added to 0.5 mg/ml Tat peptide solution in 100 mM sodium bicarbonate (pH 9.3). After incubation for 4 h at room temperature in the dark, 1 M ethanolamine was added to inactivate the residual FITC. The reaction mixture was subjected to gel-filtration column chromatography using a 12 cm Sephadex G-50 to remove the unconjugated dye [13]. The conjugation between FITC and Tat peptide was verified by an intense fluorescent band under a UV light on an SDS–polyacrylamide gel.

Confocal laser scanning microscopy. Cellular distribution of Tat peptide in *T. beigelii* cells was analyzed by confocal laser scanning microscopy. *T. beigelii* cells were incubated in YPD medium at 28 °C for 12 h, harvested, and washed with ice-cold PBS buffer. After washing the cells, they were treated with FITC-labeled Tat peptide and incubated at 30 °C for 2 min. Visualization and localization of the labeled peptide were performed by Laser Scanning Spectral Confocal Microscope (Leica TCS SP2, Leica, Swiss).

Preparation of small unilamellar vesicles. Small unilamellar vesicles (SUVs) were prepared by drying phosphatidylcholine (PC)/cholesterol (10:1, w/w) under nitrogen, suspending 100 mM of the film in 50 mM of phosphate buffer at pH 7.5, mixing by vortex, and sonicating using the tip of an ultrasonic probe. A drop was deposited on a carbon-coated grid and negatively stained with 2% uranyl acetate. Specimens were examined in a Tecnai 12 (Philips, USA) at an accelerating voltage of 120 kV.

Flow cytometry analysis. For analysis of the intracellular integrity after peptide treatment, *T. beigelii* cells (2×10^5 cells in YPD media) were first harvested at the log phase and mixed with Tat peptide at a concentration of 20 μM. The cells were incubated for a further 30 min at a physiological temperature of 28 °C under constant shaking (140 rpm) in the absence or presence of NaN_3, NaCl, and $MgCl_2$, respectively [17]. After incubation, extracellular Tat peptide was removed through excessive washing with ice-cold PBS. The fluorescence of FITC was monitored in the FL2-H channel. Flow cytometric analysis was performed by the FACScalibur flow cytometer (Becton–Dickinson, San Jose, CA).

Cell cycle analysis. Log-phased cells of *C. albicans* (1×10^9 cells) were incubated in fresh YPD containing 30 μM Tat peptide for 6 h, and washed with PBS buffer and fixed with 70% ethanol (in PBS) at 4 °C overnight [18]. Following cell-fixation, washed cells were resuspended with 0.5 ml PBS containing 200 μg RNase A and reacted for 2 h at 37 °C. After the reaction, 0.5 ml PBS containing 50 μg propidium iodide was added to the suspension and the samples were incubated for 4 h at 4 °C in the dark. Some 15,000 cells were scored [19].

Morphological changes observed by scanning electron microscopy after peptide treatment. Candida albicans cells by periodic subculturing were maintained in liquid YPD medium at 37 °C. To induce to form a mycelium, cultures were directly supplemented with 20% fetal bovine serum [20]. The dimorphic transition in *C. albicans* was investigated from cultures containing Tat peptide, incubated at 37 °C for 48 h. The dimorphic transition to hyphal forms was detected by phase contrast light microscopy (NIKON, ECLIPSE TE300, Japan).

Results and discussion

Antifungal activity against human pathogenic fungi and hemolysis on human erythrocytes

The character of 12-mer Tat peptide (YGRKK RRQRRRD) has been studied for mammalian cells, especially HeLa cells [9,21] but it has not been reported its antibiotic activities against microorganisms. To investigate the effects of Tat peptide on human pathogenic fungal strains, we examined antifungal activity of Tat peptide as compared with that of antimicrobial peptide melittin. Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2) used as a positive control is a 26-amino acid peptide that is a components of the venom from honey bee and has been known for a potent antimicrobial activity and cytotoxic activity against mammalian cells [22]. The fungal strains tested are susceptible to Tat peptide with MIC values in the 3–24 μM range (Table 1) and antifungal activity of Tat peptide is less potent than that of melittin, which determined at the MIC value of 0.44 μM. It is thought that a remarkable contrast of antifungal activity between Tat peptide and melittin may be caused by C-terminal amidation found in many antimicrobial peptide sequences which is synthesized for innate immune system and isolated from various organisms [23] with its potent cytolytic activity that could be exhibited by itself as one of the component of venom [22]. To examine the cytotoxicity of Tat peptide, we determined the hemolysis of the human Red Blood Cells (RBCs). Contrary to potent hemolytic activity of melittin,

Table 1
Antifungal activity of Tat peptide against human pathogenic fungi

Fungal strains	Peptides MIC (μM)	
	Tat peptide	Melittin
S. cerevisiae	24	0.44
T. beigelii	24	0.44
C. albicans	24	0.44
M. furfur	3	0.44

Tat peptide shows no cytotoxicity on human RBCs in all the tested concentrations (Table 2). These results demonstrated that Tat peptide shows antifungal activity that exhibits the effects on the growth of pathogenic fungal cells like established antimicrobial peptides, and no hemolytic activity on human RBCs.

Table 2
Hemolytic activity of Tat peptide

Peptides	% Hemolysis (μM)				
	25	12.5	6.25	3.13	1.56
Tat peptide	0	0	0	0	0
Melittin	100	100	100	100	63

The hemolytic activity of the peptides was evaluated by determining the hemoglobin release of 4% (v/v) suspensions of fresh human erythrocytes at 414 nm. The hemolysis percentage was calculated using the following equation: percentage hemolysis = [($Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100 − $Abs_{414\ nm}$ in PBS)]100.

Intracellular localization of Tat peptide with T. beigelii cells and the effect on the morphology of artificial liposome

To investigate the localization of Tat peptide in fungal cells, the cellular distribution of FITC-labeled peptide was visualized under confocal laser microscope. *Trichosporon beigelii*, formerly known as *Trichosporon cutaneum*, is a generally non-pathogenic fungus which is a common skin contaminant and causes innocuous infections, such as white piedra and onychomycosis, in immunocompetent hosts and has occasionally been described as causing systemic infections with a high mortality in patients with predisposing conditions [24,25]. When treated Tat peptide to *T. beigelii* cells, this peptide penetrated through the cell membrane and mainly was distributed in the fungal cell rapidly (Fig. 1A). We also investigated the intracellular internalization of Tat peptide by time or temperature to understand the localization for its antifungal activity. The staining with the nuclear-staining dye CYTO59 red fluorescent clearly indicates that Tat peptide rapidly accumulated the nuclei (Fig. 1B). Moreover, we confirmed that Tat peptide penetrates into the nuclei of *T. beigelii* cells and did not give any effect by temperature (0, 30, and 37 °C) and time (5 s–5 min) (data not shown).

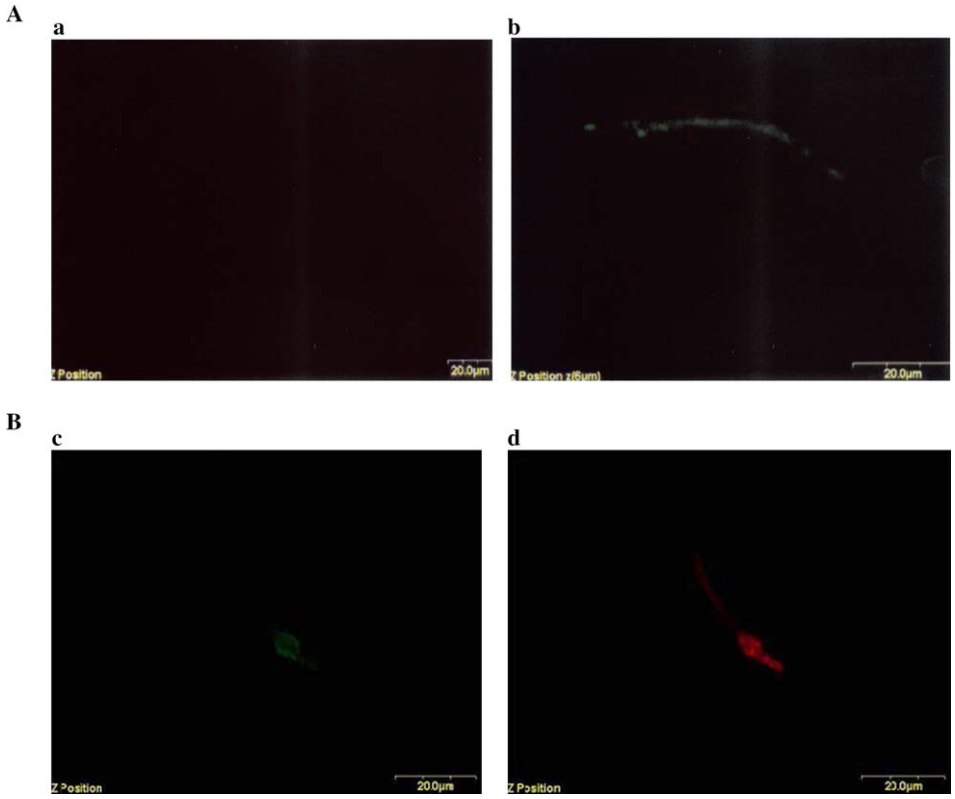

Fig. 1. Confocal fluorescence microscopy of *T. beigelii* cells treated with FITC-labeled peptide. Cells treated with FITC-labeled Tat peptide were incubated for 5 s at 28 °C. (A) a, Not treated peptide; b, FITC-labeled peptide. The intracellular localization of the peptide was examined under various condition. (B) c, FITC-labeled peptide; d, the nuclear-staining dye CYTO59 for 2 min at 30 °C.

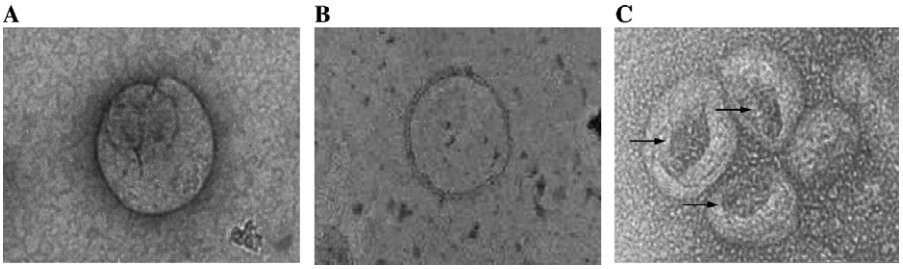

Fig. 2. Electron micrographs of negatively stained SUV composed of PC/cholesterol (10:1, w/w) in the absence (A), or in the presence of Tat peptide (B) or melittin (C).

In general, cationic antimicrobial peptides target microbial plasma membrane and disrupt cell structure by pore-forming activity after charged-interaction with plasma membrane [26]. To investigate whether Tat peptide disrupts fungal plasma membrane when this peptide penetrates through the fungal cell membrane, membrane-disrupting activity by using artificial liposome was tested (Fig. 2). Artificial Small Unilamellar Vesicles (SUVs) (PC/cholesterol; 10:1, w/w) were used as model membrane systems [27]. Melittin has been known to exhibit its antimicrobial activity through pore forming on the plasma membrane [22], and we visualized the pore-forming activity of melittin. However, the liposome treated with Tat peptide was not disrupted like the liposome not treated. These results suggest that Tat peptide has a novel mode of action for its antifungal activity, unlike the established antimicrobial peptides disrupting the cell membrane structure.

The effects of sodium azide or cations on antifungal activity of Tat peptide

In this study, we examined whether membrane depolarization or salts give some effects to antifungal activity of Tat peptide. To investigate energy-dependence on peptide uptake for *T. beigelii* cells, we treated FITC-labeled Tat peptide in the absence or presence of sodium azide (NaN_3) which is a metabolic inhibitor blocking ATP synthesis in mitochondria [28]. ATP depletion that causes various changes in cell membranes by the regulation of intracellular pH [29] exhibited scarcely any effect on the uptake of Tat peptide. This result suggests that penetration pathway of Tat peptide is energy-independent process, which is not affected by the change of cellular physiological condition through ATP depletion (Figs. 3B and C).

Additionally, the cation influence on the uptake of Tat peptide was investigated using a flow cytometry in the presence of NaCl and $MgCl_2$. In the recent reports, the MICs of cationic antimicrobial peptide were significantly increased in the presence of salt ions [30], because it caused by salt antagonism on interaction of the peptides with microbial plasma membrane. We tested salt resistance of Tat peptide at 20 mM, 150 mM NaCl and 1 mM, 5 mM $MgCl_2$; the NaCl concentration in the epithelial cell secretions of a cystic fibrosis patient is over 100 mM [31] and the $MgCl_2$ concentration was modeled using the serum divalent cation concentration of 1 mM Mg^{2+} [30]. There was no decrease in the uptake of Tat peptide in the presence of 20 mM, 150 mM NaCl and 1 mM $MgCl_2$ (Figs. 3D–F) and the peptide uptake was slightly decreased in the presence of 5 mM $MgCl_2$ (Fig. 3G). This exhibits the salt-resistance of Tat peptide on the penetrating into the fungal plasma membrane to exert its activity. These results indicate that the activity of Tat peptide was not affected by cellular energy system and various salt conditions.

The effect of Tat peptide on the cell cycle progress of C. albicans cells

Recent studies reported that cell-penetrating peptides such as PR-39 bound tightly to nucleic acids and inhibited the macromolecular synthesis of the cell [6]. To understand how Tat peptide inhibits the growth of fungal cells after penetrating into the nucleus, we investigated the effects of Tat peptide on the cellular function in the nucleus of *C. albicans* cells by performing the cell cycle analysis through determining the DNA content of cells with propidium iodide (PI), which is known for DNA-intercalating agent, with FACscalibur flow cytometry. *C. albicans*, which induces serious fungal infections leading to candidiasis, is one strain of *Candida* species that have become the fourth leading cause of bloodstream infections and carry significant morbidity and mortality [32]. After addition of Tat peptide to culture medium, *C. albicans* cells were delayed moving from G1 phase to S phase within 90 min (Fig. 4). This indicates the physiological changes induced by Tat peptide after penetration of the cell membranes, resulting in the cell death. Although more tests are needed to clarify the targets of Tat peptide in the nucleus, the cationic property of the peptide that is similar to the cationic peptide binding the nucleic acids [5] leads us to believe that this peptide inhibits some functions in the nucleus by binding to anionic materials such as nucleic acids. The result suggests that Tat peptide gives some effects on the physiology of fungal cells and it is thought that the effects are the cause leading to the fungal cell death by arrest of cell cycle progress.

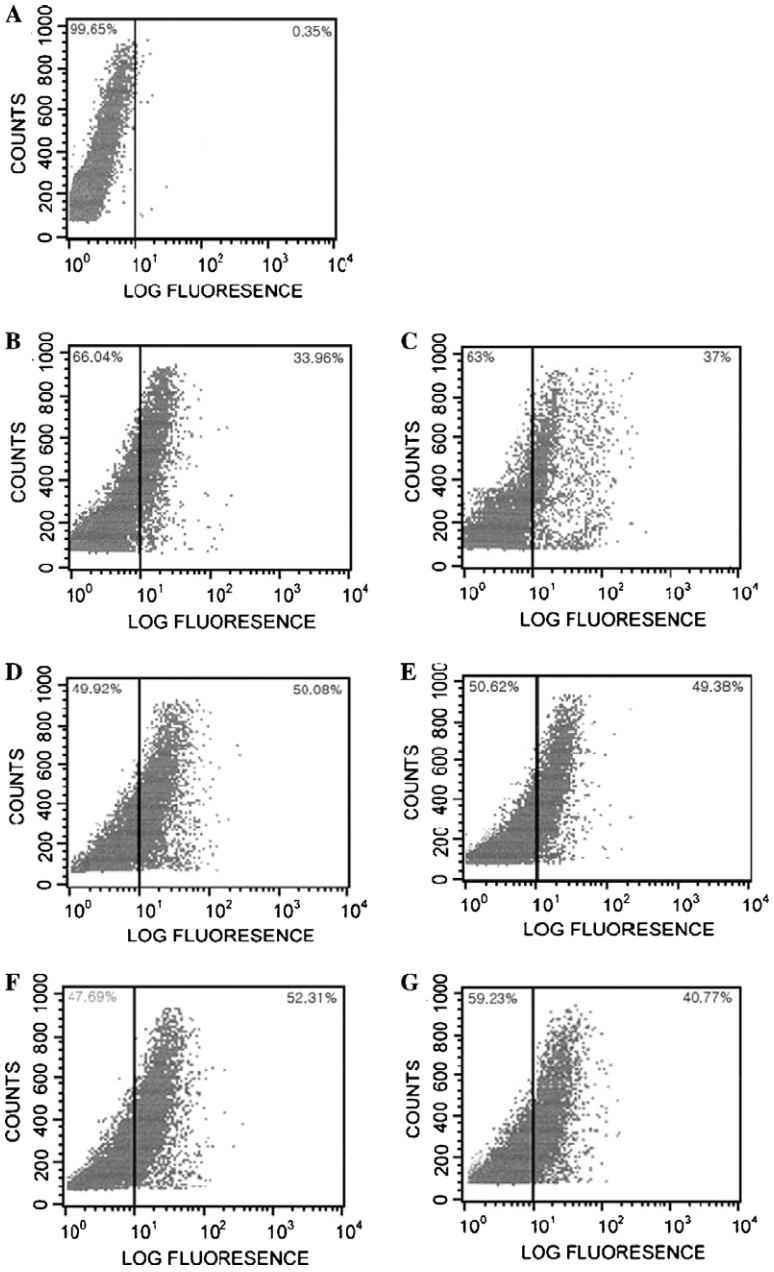

Fig. 3. The effect of sodium azide (NaN_3) and salt on activity of Tat peptide. Subcultured *T. beigelii* cells were treated with Tat peptide labeled with FITC. The cell population showing fluorescence was analyzed by the FACScalibur flow cytometry and the increments of the log fluorescence signal represent uptake of peptide labeled with FITC: (A) control, (B) Tat peptide-treated cells, (C) Tat peptide- and 0.002% NaN_3-treated cells, (D) Tat peptide- and 20 mM NaCl-treated cells, (E) Tat peptide- and 150 mM NaCl-treated cells, (F) Tat peptide- and 1 mM $MgCl_2$-treated cells, and (G) Tat peptide- and 5 mM $MgCl_2$.

The effect of Tat peptide on dimorphic transition of C. albicans

The ability of some fungal strain(s) to undergo a morphological transition between unicellular forms and hyphae structures may be considered a simple model of cellular development [33]. *C. albicans* is prototypic dimorphic yeast and this diploid pathogen is of increasing importance in human medicine. In *C. albicans*, dimorphism plays a crucial role in pathogenesis, with mycelial shapes being predominantly found during a host tissue invasion [34]. The applications of some factor to induce dimorphic transition in *C. albicans* have been reported. To induce mycelial form in this test, 20% fetal bovine serum (FBS) directly supplemented to culture of *C. albicans* cells. In order to investigate the effect of Tat peptide on the dimorphic transition of *C. albicans*, it tested in cultures containing 10 or 20 μg Tat peptide and incubated with FBS at 37 °C for 48 h (Fig. 5). Tat peptide inhibited and destroyed the mycelial forms of *C. albicans* cells, indicating that this peptide could be applied to design a synthetic peptide for therapeutic

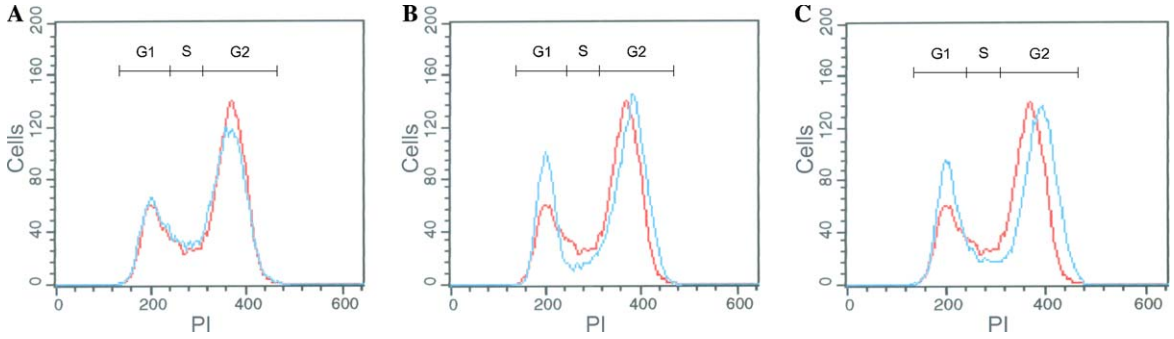

Fig. 4. Inhibition of the cell cycle progress for *C. albicans* cells by Tat peptide. The cell cycle progress of fungal cells was examined by PI-stained DNA contents by FACScalibur. Some 15,000 fungal cells were scored after incubation for 30 min (A), 60 min (B), and 90 min (C). Red, not treated with the peptide; blue, Tat peptide-treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

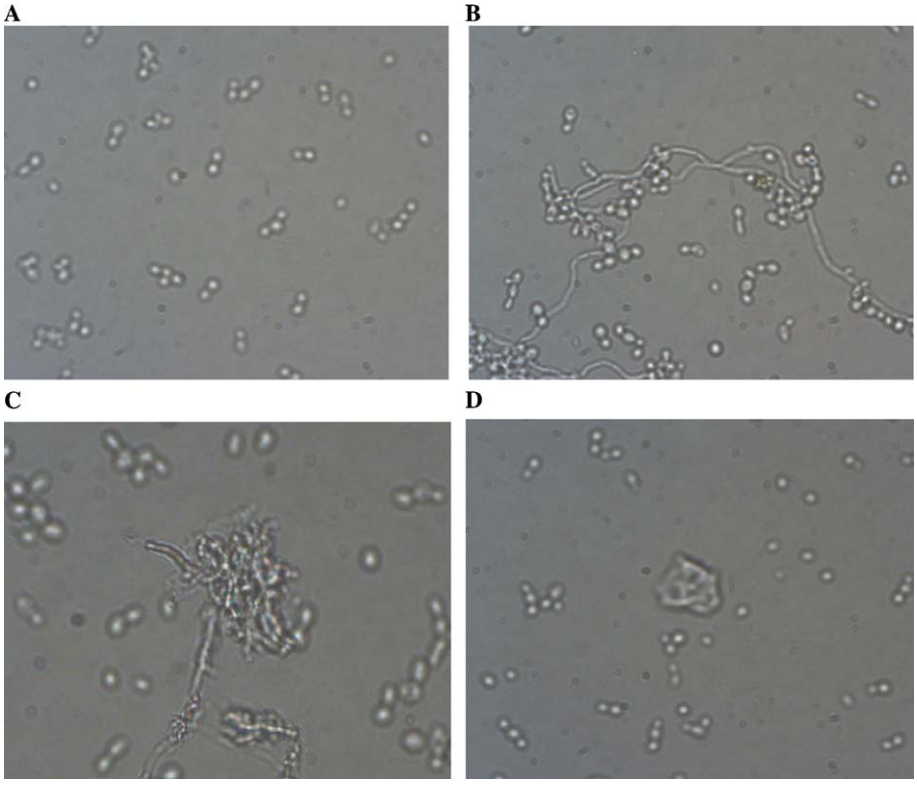

Fig. 5. The effect of Tat peptide on the dimorphic transition in *C. albicans*. To induce hyphal form, the cultures were directly supplemented with 20% fetal bovine serum (FBS) and each culture was incubated with various concentrations of Tat peptides (0, 10, and 20 μM) at 37 °C for 48 h in YPD media. Yeast control no treated with the peptides and FBS (A), and (B–D) fully.

agent to treat the infectious diseases by the invasion of *C. albicans* cells in human body.

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