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RESEARCH PAPER

Cell Penetrating Peptides as Efficient Nanocarriers for Delivery of Antifungal Compound, Natamycin for the Treatment of Fungal Keratitis

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ABSTRACT

Purpose Enhancing the penetration ability of the antifungal drug natamycin, known to possess poor penetration ability through the corneal epithelium, by complexing with cell penetrating peptides.

Methods The drug, natamycin was conjugated to a cell penetrating peptide, Tat-dimer (Tat_2). The uptake ability of the conjugate in human corneal epithelial cells and its antifungal activity against filamentous fungi, *F.solani* has been elucidated.

Results The cellular penetration ability of natamycin increased upon conjugation with Tat_2. The conjugation between natamycin and Tat_2 also lead to enhanced solubility of the drug in aqueous medium. The antifungal activity of the conjugate increased two-folds in comparison to unconjugated natamycin against clinical isolates of *F.solani*.

Conclusion The formation of CPP-natamycin complex is clinically significant as it may enhance the bioavailability of natamycin in corneal tissues and aid in efficient management of fungal keratitis.

KEYWORDS antifungal activity · cell penetrating peptide · corneal tissue · drug delivery · solubility

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ABBREVIATIONS

CPP	Cell penetrating peptide
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
IV	Intravenous
MIC	Minimum inhibitory concentration

INTRODUCTION

Fungal keratitis or inflammation of the cornea due to fungal infection is a common eye infection. It accounts for more than 50% of the cases of microbial keratitis especially in the tropical and subtropical regions with a high incidence of occurrence in countries such as India (44%) and Ghana (38%) [1–4]. In a study published in 2002, it was found that the occurrence of fungal keratitis was gender dependent due to differences in occupational preferences, with a male-to-female ratio being 2.5:1 [5]. More than 25% of the cases were reported by people working in the agricultural sector followed by manual labourers. (20%) [5]. In a study by Gopinathan *et al.*, trauma was the commonest predisposing factor (81.9%) for the occurrence of fungal keratitis [6].

Fungal keratitis may lead to complications such as corneal perforation and has been reported to be a major cause of monocular blindness (blindness in the affected eye) in the developing countries [7]. The most common causative agents of fungi-associated keratitis are the filamentous species *Fusarium* (37–62%) and *Aspergillus* (24–30%) worldwide, with *Fusarium* being the predominant pathogen in India [1, 5, 8]. In a study from East Africa, it has been reported that more than half of the cases of culture positive microbial keratitis were due to filamentous fungi [9]. In a study from U.S., 78% cases of fungal keratitis following ocular trauma and 86% cases following refractive contact lens wear, were caused by filamentous fungi [10]. It has been reported that *Fusarium* associated keratitis is

more severe and less amenable to therapy due to its rapidly progressive nature as compared to *Aspergillus* keratitis [1].

The first line of FDA approved treatment for fungal keratitis is a 5% sterile suspension of natamycin [11, 12]. The drug natamycin is administered topically and has a broad spectrum activity against filamentous fungi, therefore, it is considered as the drug of choice for the treatment of filamentous fungi-associated keratitis. However, natamycin shows poor absorption upon topical application since the corneal epithelium serves as a barrier for its internalization [13]. Due to the poor intraocular penetration ability of natamycin coupled with high tissue binding, its bioavailability reduces in the corneal tissue. Hence, the corneal epithelium needs to be debrided while administering natamycin [14, 15]. The poor penetration of natamycin also limits its use as an effective treatment for deep stromal fungal keratitis where fungi affects the deeper layers of cornea [1]. Also, it is noteworthy that natamycin can be administered only as a topical suspension and by no other route as natamycin *per se* is not soluble in water [16].

Other available antifungal drugs such as amphotericin B, voriconazole, fluconzole and flucytosine, although efficacious, have been reported to exhibit systemic toxicity [1]. In a global survey, 80% ophthalmologists treating fungal keratitis, suggested that the existing treatments for fungal keratitis are only moderately effective in curing the fungal infections of the cornea [13]. Reports have also shown that the resolution of fungal infection with topical medication occurred in only 7.6% cases with 92.4% requiring surgical intervention [17]. Another disadvantage of the current topical medications involves repeated administration of the drug for prolonged duration that increases the overall cost of treatment for the patient.

Therefore, efficient ways to deliver the antifungal compounds topically, particularly natamycin, needs to be developed. Enhancement of the penetration ability of natamycin through the corneal epithelium is clinically significant as it will increase the bioavailability of the drug. Effective topical therapy may also decrease the incidence of eye threatening complications leading to better treatment outcome and less need for surgical interventions. Recently, a nanoparticle based formulation using Dex-b-PLA nanoparticles for encapsulating natamycin has been used in combination with model contact lens material to increase the release and uptake of natamycin to the eye tissue [18]. However, the problem of poor penetration of natamycin through the corneal epithelium remains unsolved in the above approach since PLA-nanoparticles are not efficient in membrane translocation [19, 20]. They have been shown to be used in conjunction with other cell permeable agents for improved cellular translocation ability [20, 21].

Hence, in the present study an attempt has been made to enhance the penetration ability of the widely used antifungal compound, natamycin by conjugating it to a cell penetrating peptide. Cell penetrating peptides (CPPs) have been chosen as the delivery vehicle because of their ability to translocate across the cell membrane without causing cell death [22, 23]. They are usually cationic in nature and have been shown to carry vast range of cargo molecules such as oligonucleotides (DNA, siRNA), protein molecules (β-galactosidase), quantum dots and drug molecules [22, 24–26]. They possess advantages such as low risk of cytotoxicity, oncogenicity and an ability to cross the blood–brain barrier [27]. Recent reports have also demonstrated the trans-dermal penetration of cell penetrating peptides conjugated with various macromolecules [28–31].

To the best of our knowledge, the present study for the first time demonstrates that the penetration and solubility of natamycin *per se* enhanced by conjugation with a cell penetrating peptide.

MATERIALS AND METHODS

Synthesis of Peptide-Natamycin Complex

The peptides Tat_2 (RKKRRQRRRRKKRRQRRR-NH_2) and $MTat_2$ (AKKRRQRRRAKKRRQRRR-NH_2) were custom synthesised using f-moc-rink amide linker (solid phase synthesis) and labelled fluorescently with FITC at the N-terminus (GenPro Biotech Pvt. Ltd., India). Natamycin was linked to these peptides covalently. An amide linkage was formed between the primary amine of natamycin and carboxy terminus of the peptide (Fig. 1). The peptide drug conjugate (Tat_2-Nat (RKKRRQRRRRKKRRQRRR-natamycin) and $MTat_2$-Nat (AKKRRQRRRAKKRRQRRR-natamycin)) thus formed were labelled with FITC for reporting the cellular uptake of the conjugate in the cell. The stoichiometric ratio of $MTat_2$/Tat_2: natamycin was maintained at 1:1 for each conjugate. The unconjugated and conjugated peptides were purified by HPLC followed by mass spectroscopic analysis for molecular weight determination. Natamycin alone was also labelled with FITC as a control.

Cell Culture

HeLa cells were obtained from National Centre for Cell Science, Pune, India. They were cultured in DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin (Cell Clone, Genetix Biotech Asia Pvt. Ltd., India) and incubated at 37°C, 5% CO_2.

A primary culture of human corneal epithelial cells (HCE) was kindly provided by Prof. PK Roy Chaudhary, Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, India. HCE cells were cultured in DMEM: F12 (1:1) with 10% FBS, 2% pencillin-streptomycin at 37°C in

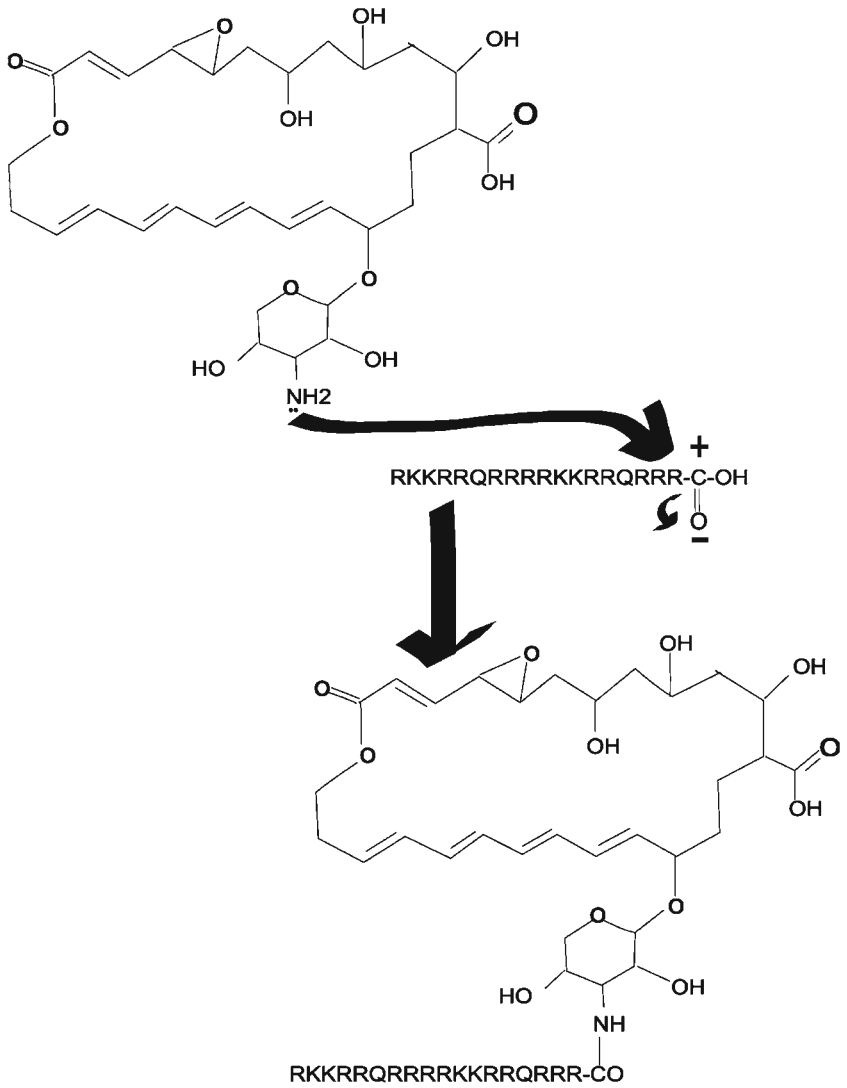

Fig. 1 Strategy of formation of Tat_2-Nat complex.

an atmosphere of 5% CO_2 (Cell Clone, Genetix Biotech Asia Pvt. Ltd., India).

TRANSLOCATION OF PEPTIDE-DRUG COMPLEX IN MAMMALIAN CELLS

Internalization of Peptide-Drug Complex

For initial assessment of uptake ability of the conjugated product, HeLa cells were employed. Cells were seeded at a density of $2X10^5$ cells/well in a 24-well plate one-day prior to conducting the experiment. Cells were equilibrated with serum-free medium for 30 min followed by incubation with 5 μM of FITC labelled natamycin, $MTat_2$, Tat_2, $MTat_2$-natamycin and Tat_2-natamycin for 1 h at 37°C, 5% CO_2. The cells were washed thrice with 1X PBS (pH 7.4) followed by visualization under the microscope and flow cytometry. Similar procedure was employed to study internalization in HCE cells.

Fluorescence Microscopy

Uptake of the unconjugated and conjugated peptides/drugs in mammalian cells and fungal hyphae was visualized using epifluorescence microscope (Olympus IX51, excitation/emission 495/510 nm).

Confocal Laser Scanning Microscopy

HeLa and HCE cells were seeded on glass coverslips at a density of 2×10^6 cells/well overnight. The cells were incubated with peptides as mentioned above. The cells were washed thoroughly with PBS followed by visualization under the

confocal microscope (Olympus Fluoview 1000; excitation/emission 488 nm/510 nm). The images were taken at 40X magnification with 3X zoom. Z-stacking for natamycin and Tat_2-natamycin treated cells was performed to compare the extent of uptake in the cells. Forty frames were recorded for Tat_2-Nat and fifteen frames were recorded for natamycin alone.

Flow Cytometry

The cells (HeLa and HCE) were trypsinized with 0.25% Trypsin-EDTA solution (Cell Clone, Genetix Biotech Asia Pvt. Ltd., India) and washed twice with PBS. The cell pellet was resuspended in sheath fluid and run in flow cytometer (BD FACSAria III, Becton Dickinson, USA). The data was analysed using FACSDiva ver 6.0 software. Live cell population was gated using forward and side scatter plots, 10,000 events were recorded per sample with three replicates.

MTT Assay

The cytotoxicity of unconjugated and conjugated natamycin and peptides in mammalian cells was assessed using MTT assay. Cells were seeded in a 96-well plate at a density of $2X10^3$ cells/well. Incubation with the peptide was made at concentrations as mentioned in the above section. MTT reagent (Sigma Aldrich) was added at a concentration of 1 mg/ml and incubated at 37°C, 5% CO_2 for 2-4 h. DMSO was added to the wells and plates were read at 570 nm (MultiSkan Go, ThermoScientific, India).

Assessment of Cell Toxicity on Corneal Endothelial Cells

Human donor corneal tissues, preserved in Optisol GS medium (Bausch & Lomb, USA) were obtained from VNH Eye Bank, Mumbai, India and Ramayamma International Eye Bank, Hyderabad, India. The baseline endothelial cell counts for all the tissues were obtained using Eye Bank Specular Microscope EKA - 10 (Konan Medical, INC. Japan). Corneal tissues were divided into four groups, Group 1 (control group) received no drug while groups 2, 3 and 4 were preserved at 4°C for 48 h in the presence of 5 μM each of FITC-$MTat_2$-Nat, FITC-natamycin and FITC-Tat_2-Nat, respectively, added to Optisol GS medium. Endothelial cell counts were obtained 48 h post preservation at 4°C with the peptides. The effect of peptide-natamycin complex on endothelial cells in the corneal tissue was assessed on two parameters: cell count and hexagonality. The data obtained was statistically validated using a two-way ANOVA model.

Mechanism of Uptake of Peptide-Natamycin Complex

HCE cells were treated with endocytic inhibitors cytochalasin D (10 μM), chlorpromazine hydrochloride (28 μM) and 4°C for 30 min followed by addition of 5 μM of natamycin, $MTat_2$, Tat_2, $MTat_2$-Nat and Tat_2-Nat. The cells were incubated with the peptides for 1 h followed by washing with 1X PBS and visualization under epifluorescence microscope. For quantitative assessment flow cytometry was carried out.

STUDY OF ANTIFUNGAL ACTIVITY OF PEPTIDE-DRUG COMPLEX

Microbroth Dilution Assay for Assessing Antifungal Activity of the Peptides

The antifungal activity of the unconjugated natamycin and peptide-natamycin complex was assessed on *Fusarium solani* (MTCC No. 10158) using two-fold microbroth dilution assay as per CLSI standards [32]. Briefly, the fungus was grown on ME (Malt extract) agar for 24–30 h at 30°C in dark. From the freshly prepared sporulating fungal plates, spores were harvested in ME broth and counted on a hemocytometer. $2X10^4$ fungal spores per well were dispersed in each well with varying concentrations of unconjugated natamycin and peptide-natamycin complex (5-30 μM) in a 96-well microtiter plate. The plates were incubated for 24 h at 30°C, 150 rpm and read at 530 nm. A sterility control was maintained for every experiment to check for contamination.

Testing of Antifungal Activity of the Peptides in Fungus Infected Mammalian Cells

HCE cells were seeded at a concentration of 4×10^5 cells per well in a 24-well plate. After 24 h, cells were infected with 2×10^4 spores/well of *F. solani* and incubated with 5 μM and 10 μM unconjugated natamycin and peptide-natamycin complex. The infected cells were incubated for 24 h at 37°C and observed under the light microscope. Further, a 1:10 dilution of the infected samples was plated on ME agar and colonies were counted.

Uptake of Peptide-Natamycin Complex in Fungal Hyphae

The fungal spores of *F. solani* were harvested and seeded at a concentration of $2X10^4$ spores/well in ME broth. The spores were allowed to germinate and grow for 24 h at 30°C, 150 rpm. After 24 h, the hyphae were incubated with 5 μM and 10 μM unconjugated natamycin and peptide-natamycin complex for 1 h at 30°C. The hyphal segments were visualized

under the microscope to assess the uptake of peptide-natamycin complex and unconjugated natamycin.

RESULTS

Formation of Natamycin-Peptide Complex

Natamycin was covalently linked to cell penetrating peptides $MTat_2$ and Tat_2 successfully using amide linkage. Natamycin, a polyene antibiotic does not dissolve in water and is sparingly soluble in methanol [33]. A colloidal suspension of the drug is obtained in organic solvents such as DMSO. It was observed that upon conjugating natamycin to CPPs (Tat_2 and $MTat_2$), the solubility of the drug enhanced 100-fold in water, forming a clear solution.

TRANSLOCATION OF PEPTIDE-DRUG COMPLEX IN MAMMALIAN CELLS

Internalization of the Peptide-Drug Complex in HeLa and HCE Cells

For an initial assessment of cell penetration activity of peptide-natamycin complex, HeLa cells were employed. The activity of the complex was also tested by incubating HCE cells with FITC-natamycin and FITC-Tat_2-Nat. It was observed that both natamycin and Tat_2-Nat were able to internalize in both HeLa (data not shown) and HCE cells (Fig. 2). However, Z-stacking revealed that Tat_2-Nat was able to internalize efficiently inside the cells, primarily localizing in the nucleus and partly in the cytoplasm while FITC-natamycin remained bound to the membrane and did not internalize inside the cells (**Movie 1 and 2**). $MTat_2$-Nat showed similar uptake profile as natamycin while unconjugated $MTat_2$ and Tat_2 displayed 1.5-fold higher uptake in cells as compared to $MTat_2$-Nat and Tat_2-Nat.

The flow cytometry data confirmed the uptake behaviour as observed under the microscope (Fig. 3). It was also observed that Tat_2-Nat did not cause any significant toxicity to HCE cells with viability being maintained at >90% comparable to cells with no treatment (Fig. 4).

Effect of Peptide-Natamycin Complex on Corneal Endothelium

Twelve human donor corneal tissues obtained from eye banks were divided into four groups of three corneas each. It was observed that preservation with either natamycin alone or peptide-natamycin complex did not affect the cell count significantly after 48 h of preservation with respect to control (p-value = 0.93, $\alpha = 0.05$). Similarly, the hexagonality of the cells was not significantly affected upon incubation with peptide-natamycin complex for 48 h with respect to control (p-value = 0.68, $\alpha = 0.05$; Fig. 5).

Mechanism of Uptake

To further understand the mechanism of uptake of peptide-natamycin complex in HCE cells, various endocytic inhibitors such as cytochalasin D (10 μM), chlorpromazine hydrochloride (28 μM) and low temperature (4°C) were used. It was found that the uptake of natamycin, $MTat_2$-Nat and Tat_2-Nat reduced in cells treated with endocytic inhibitors as compared to control cells. The uptake of natamycin reduced 1.3 times at 4°C while it remained unaffected in the presence of cytochalasin D and chlorpromazine. Likewise, the uptake of Tat_2-Nat reduced 2-folds at 4°C, 1.3-folds with cytochalasin D treatment and 4-folds with chlorpromazine. The decrease in

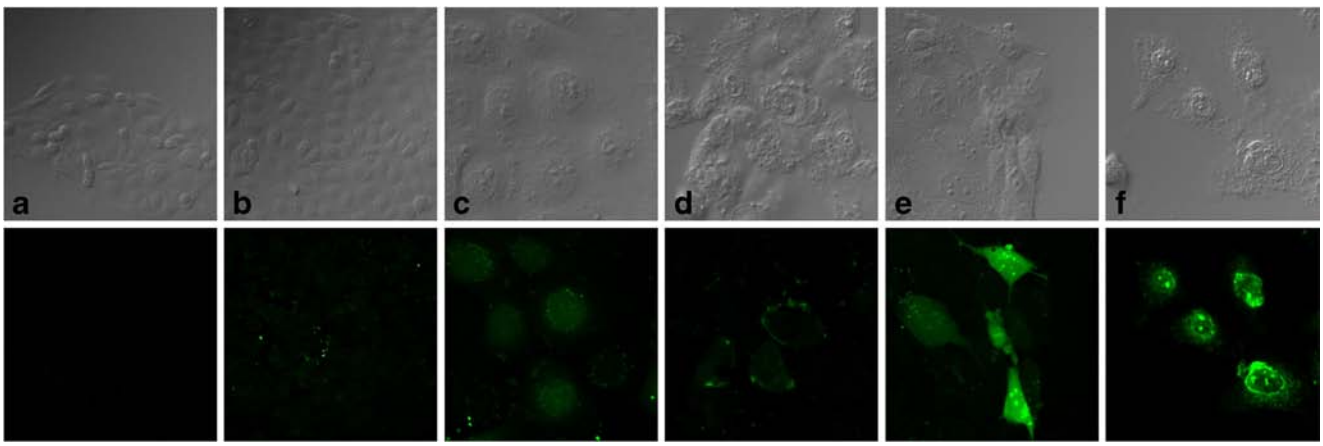

Fig. 2 Cellular translocation of natamycin-CPP complex in HCE cells as analysed by confocal microscopy (**a**) Control (**b**) Unconjugated natamycin (**c**) $MTat_2$ (**d**) $MTat_2$-Nat (**e**) Tat_2 (**f**) Tat_2-Nat. Scale bar: 20μM.

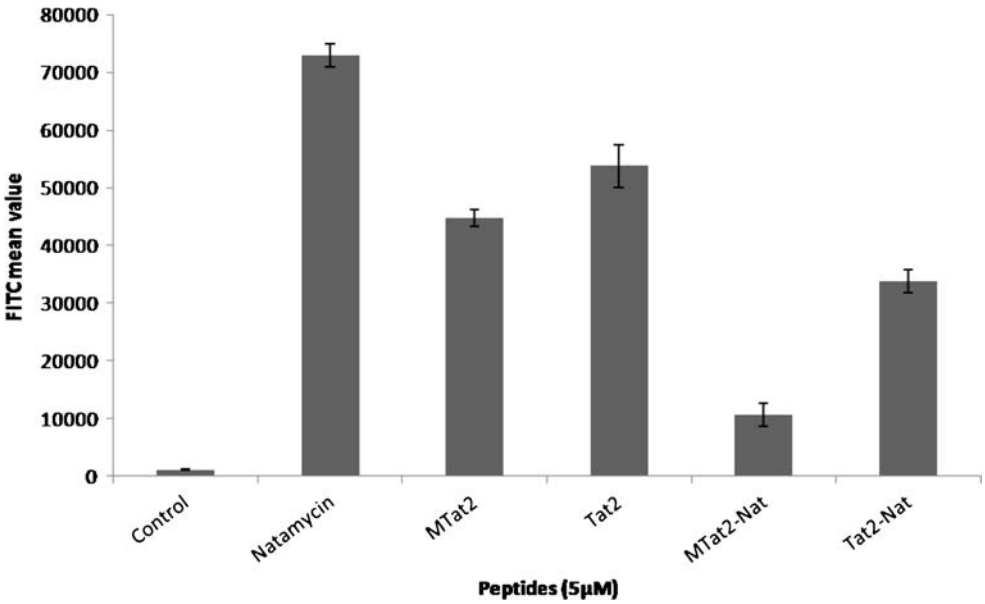

Fig. 3 Study of cellular uptake of conjugated peptides in HCE cells using flow cytometry.

uptake of $MTat_2$ and Tat_2 was 2-fold in presence of chlorpromazine though no difference could be seen at 4 °C or in the presence of cytochalasin D (Fig. 6).

Study of Antifungal Activity of Peptide-Natamycin Complex

Antifungal Activity Assessment

The micro-broth dilution assay for assessing the antifungal activity revealed that the MIC for *F. solani* with Tat_2-Nat is 3 μM. Our studies also corroborated reported MIC range for natamycin against *F. solani* as 6-12 μM [5, 34: Fig. 7]. Tat_2, $MTat_2$ and $MTat_2$-Nat also showed complete inhibition of growth of *F.solani* at 5 μM concentration.

To mimic the *in vivo* conditions, the antifungal activity of the conjugated and unconjugated natamycin was compared by infecting HCE cells with spores of *F. solani*. It was observed that the growth of the *F. solani* was completely inhibited in the presence of Tat_2-Nat and $MTat_2$-Nat at a concentration of 10 μM while natamycin alone was not able to inhibit the growth of the spores post infection (Fig. 8A and B).

Uptake of Conjugates in Fungal Hyphae

It was observed that FITC-Tat_2-Nat internalized efficiently in the hyphae of *F. solani* at a concentration of 5 μM after 1 h incubation and complete internalization in all hyphae could be seen at 10 μM. It was also found that natamycin alone

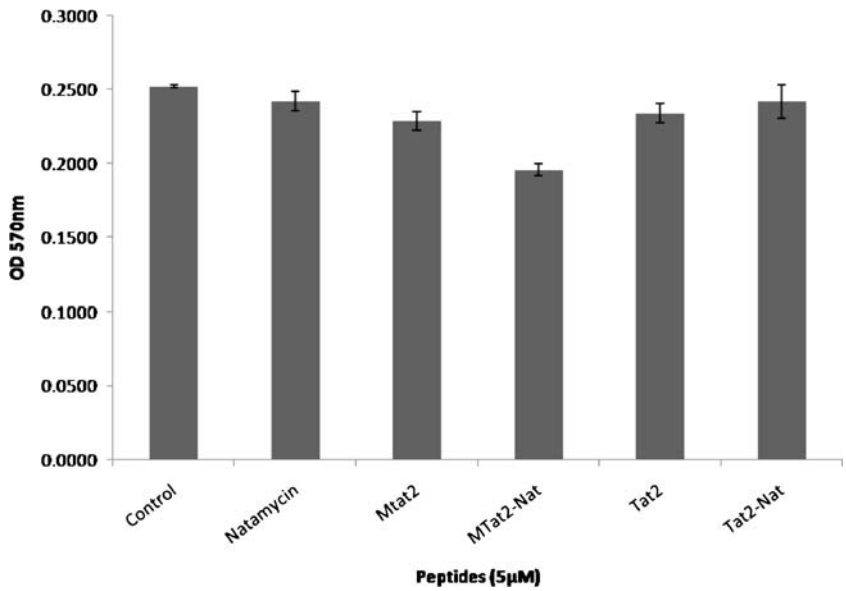

Fig. 4 MTT assay for viability assessment of HCE cells in presence of natamycin conjugated peptides.

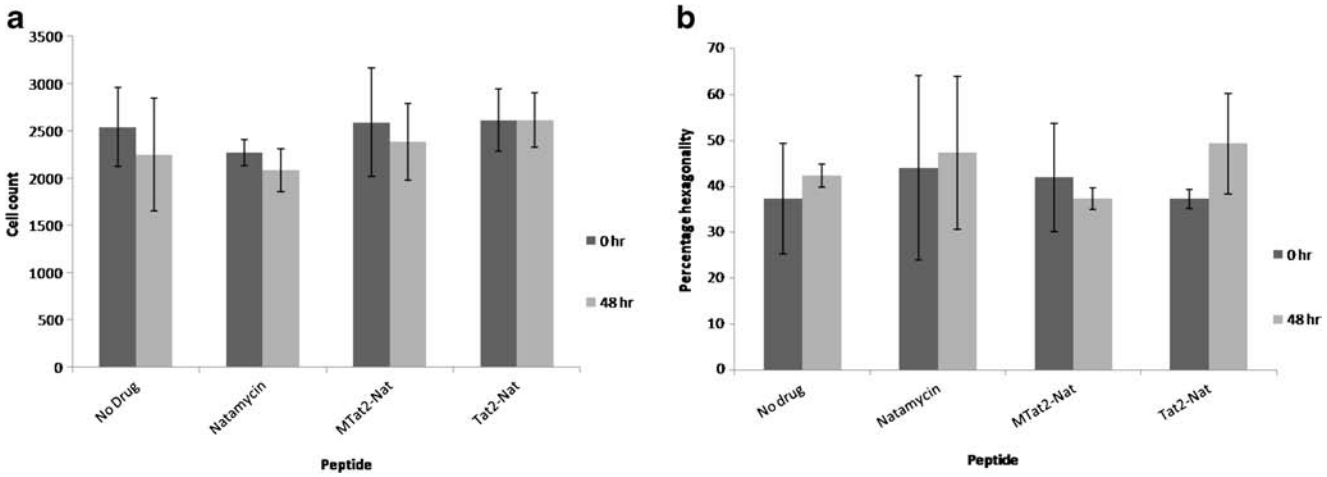

Fig. 5 Effect of peptide-natamycin complex on corneal endothelium (**a**) cell count and (**b**) hexagonality of cells using specular microscopy.

could not internalize in the hyphae of *F. solani* at either concentration (Fig. 9). $MTat_2$-Nat internalized slightly at 5 μM and 10 μM while $MTat_2$ and Tat_2 showed significant uptake in hyphae at both the concentrations.

DISCUSSION

Natamycin, a polyene antibiotic, is used as the first line of treatment against fungal keratitis because of its strong antimicrobial activity against a wide range of fungal pathogens. It can be administered topically and hence, does not cause any systemic toxicity. However, due to high tissue binding ability of natamycin and poor penetration ability across the corneal epithelium, good treatment outcomes have been noted only in mild infections [1] with dismal outcomes in moderate to severe infections [9].

Keeping this in view, an attempt has been made in the present study to increase the penetration capability of natamycin by conjugating it to cell penetrating peptide (Tat_2). The peptide Tat-dimer (Tat_2) has been employed in the present investigation because of its higher activity than Tat peptide [35]. The peptide and the drug were covalently conjugated using an amide linkage. In an initial attempt the primary amine of the Lys in the peptide sequence was conjugated to the carboxyl group of natamycin. It was observed that this type of conjugation drastically reduced the cell

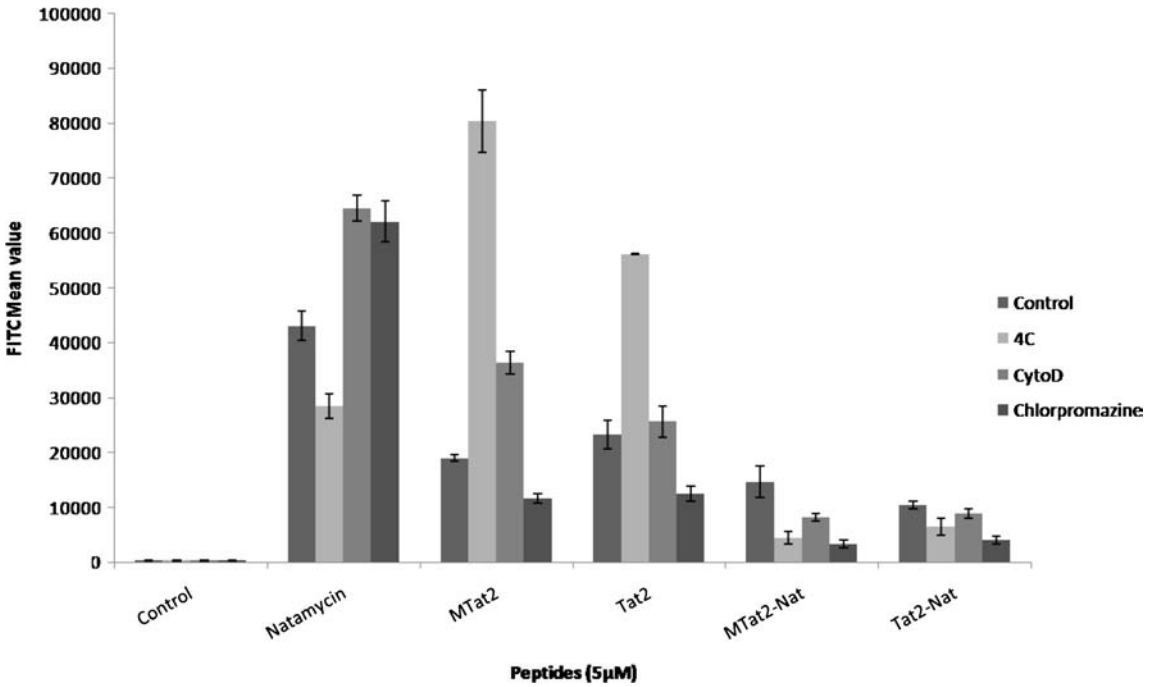

Fig. 6 Study of mechanism of uptake of peptide-natamycin complex in HCE cells in presence of endocytic inhibitors, using flow cytometry.

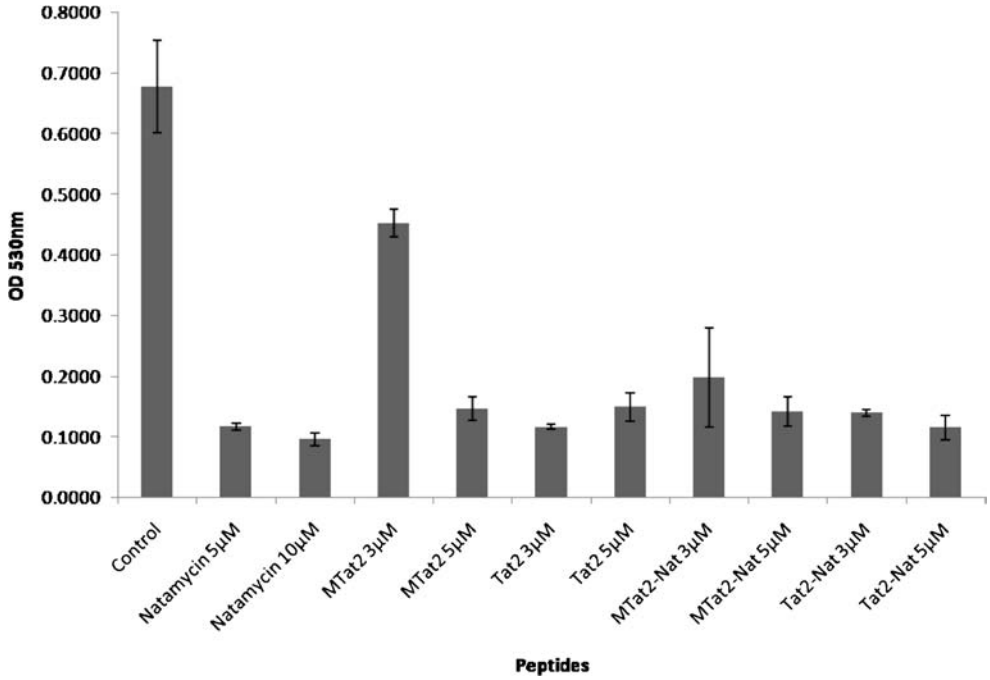

Fig. 7 Antifungal activity assessment of natamycin-peptide complex against *Fusarium solani*.

penetrating ability of the CPP as well the antifungal activity of natamycin (data not presented). Therefore, the conjugation strategy was changed and amide linkage was formed at the C-terminus of the peptide (Tat_2, $MTat_2$). By using this strategy, we were successfully able to enhance the penetration ability of natamycin.

Cells treated with natamycin gave high signals in flow cytometry even after trypsinization because natamycin is not degraded by trypsin as opposed to peptides as a result it remains bound to cell membrane emitting signals in flow cytometry. Also, because of the small size and hydrophobic nature of natamycin, it might be able to interact with the membrane and remain embedded in it giving a false positive result in terms of uptake by the cells.

Natamycin has been reported to be insoluble in aqueous solutions at physiological pH [33]. The formation of CPP-Natamycin complex not only enhanced the penetration ability of the drug but it also imparted water soluble property to the drug. The solubility to natamycin could have been provided by Tat_2, which being cationic in nature is completely soluble in aqueous environment at physiological pH. The water solubility of the drug may lead to enhanced absorption and bioavailability of the drug in the corneal tissue. Also, it may enable natamycin administration *via* alternate routes. Further studies in this regard are being carried in our lab.

The toxicity of the peptide-natamycin complex was assessed in HCE cells and corneal endothelium. It was observed that peptide-natamycin complex did not show significant toxicity on the either HCE cells or corneal endothelium tissue at 5 μM. The toxicity of peptide-natamycin complex was also tested at a range of concentrations (2.5–10 μM) on HCE cells. It was observed that no significant cellular cytotoxicity was exhibited by the peptide-natamycin complex at the tested concentrations(data not shown). The specular study data revealed that the endothelial cell counts and hexagonality of the corneal endothelium remained unaffected in the presence of the peptide-natamycin complex.

The mechanism of entry of CPPs is divided into two types: energy dependent (endocytosis) and energy independent (direct) uptake. In order to understand the mechanism by which the CPP-natamycin complex internalizes inside the cell, endocytic inhibitors were used. It was found that at 4°C, the uptake reduced 2-fold for Tat_2-Nat indicating that it is internalizing by endocytic or energy dependent process. Though, the exact endocytic process by which it enters the cell could not be elucidated since inhibition in uptake was observed for both clathrin-mediated uptake (chlorpromazine) and macropinocytosis (cytochalasin D). Also, CPPs have been reported to enter cells by more than one pathway [22], we speculate that Tat_2-Nat also enters the cells by more than one endocytic pathway.

On the other hand, in case of natamycin treated cells, cytochalasin D and chlorpromazine did not show any effect on its internalization while at 4°C, significant reduction (2-fold) could be observed. It could be speculated that natamycin was not able to interact efficiently with the cell membrane at 4°C due to reduced fluidity of the cell membrane at lower temperatures [36].

We also found that the antifungal activity of Tat_2-Nat was higher compared to unconjugated natamycin for *F. solani*. The studies were also extended to *S. schenckii* and *A. fumigatus*. The MICs with Tat_2-Nat for *S. schenckii* and *A. fumigatus* were

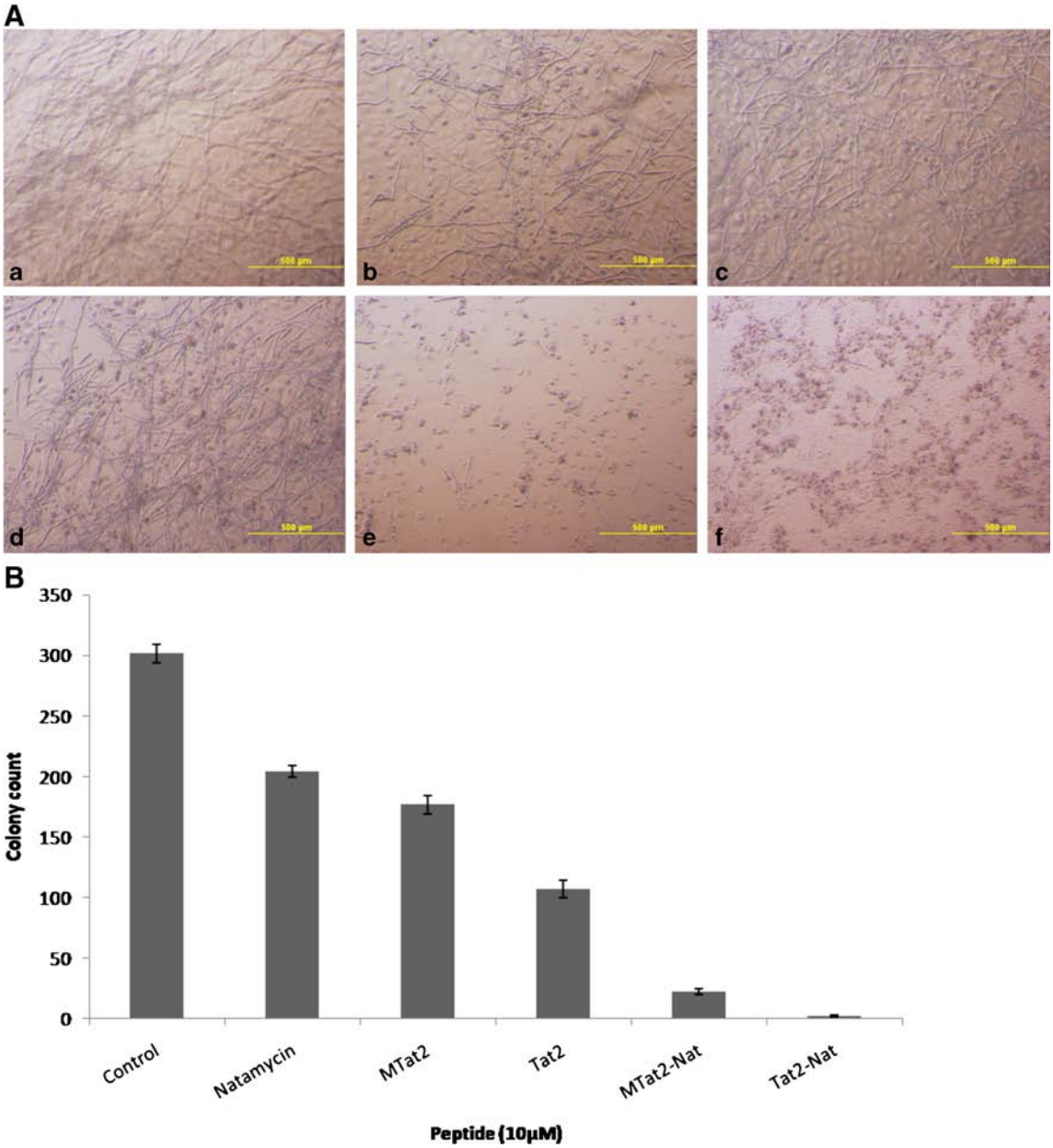

Fig. 8 (**A**) *F. solani* infected HCE cells treated with natamycin conjugated peptides (**a**) Control (**b**) Natamycin (**c**) $MTat_2$ (**d**) Tat_2 (**e**) $MTat_2$-Nat (**f**) Tat_2-Nat. (**B**) Quantitative estimation of the inhibition of growth of *F. solani* in HCE infected cells.

obtained at 20 and 30 μM respectively (data not shown). The increased antifungal activity could be due to the ability of Tat_2-Nat to penetrate into the fungal hyphae and spores. Another reason for increased antifungal activity of the conjugate complex could be that Tat_2 itself exhibits antifungal activity [Fig. 7; 37]. It has been reported that Tat, the monomer of Tat_2 also exhibits antifungal activity [38]. In the complex, the two moieties that is, peptide (Tat_2) and antifungal drug (natamycin) might be acting in synergism exhibiting better activity against fungal growth. Further studies are currently being carried out to assess the antifungal sensitivity of the conjugates on various ocular and non-ocular fungal isolates.

In order to mimic the *in vivo* condition of early stages of fungal infection in the cornea, HCE cells were infected with spores of *F.solani*. Comparative study of the antifungal activity of the unconjugated and conjugated natamycin in the above formed system revealed that Tat_2-Nat showed better inhibitory activity as compared to natamycin alone although the concentration of the complex at which it showed effect was twice that in micro-broth dilution assay. It is proposed that since the experimental set-up mimicked the *in vivo* infection conditions, losses in the availability of the complex pertaining to cellular interaction might account for requirement of higher concentration of the peptide-natamycin complex.

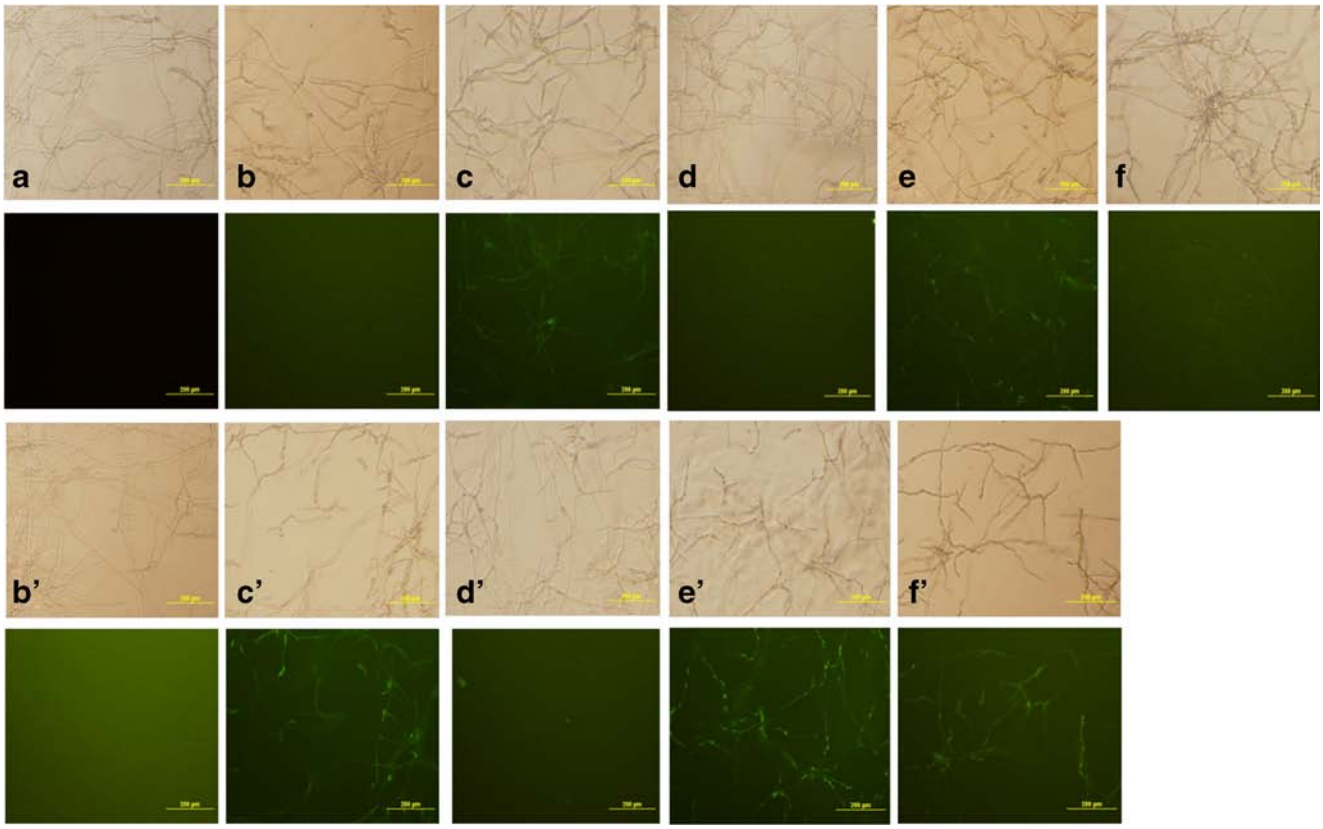

Fig. 9 Uptake of FITC labelled conjugated drug and peptide-drug complex in hyphal segments of *F. solani* (**a**) Control (**b**, **b'**) Natamycin: 5μM, 10μM (**c**, **c'**) MTat$_2$: 5μM, 10μM (**d**, **d'**) MTat$_2$-Nat: 5μM, 10μM (**e**, **e'**) Tat$_2$: 5μM, 10μM (**f**, **f'**) Tat$_2$-Nat: 5μM, 10μM.

CONCLUSION

The present study for the first time demonstrated the enhanced penetration of the well known drug natamycin by conjugating it with a cell penetrating peptide. The strategy may play an effective role in fungal keratitis management. Interestingly, the solubility of the peptide-drug complex also increased, thereby, making it more amenable for developing efficient clinical formulations for better outcomes in the treatment of severe cases of fungal keratitis. Also, the current strategy can be extended to other antifungal agents such as amphotericin B. The developed conjugate may be clinically significant as better absorption of the drug may help in better bioavailability leading to better efficacy and administration of lesser doses to the patient. However, further elucidation of its performance *in vivo* will establish its significance over the existing line of treatment against fungal keratitis.

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