Article ID: 0253-2778(2009)04-0344-07

Transdermal delivery of fusion green fluorescent protein mediated by covalently associated TD1 peptide

SUN Zheng, WEN Long-ping

(*Hefei National Laboratory for Physical Sciences at Microscale, and School of Life Sciences, University of Science and Technology of China, Hefei* 230027, *China*)

Abstract: TD1 peptide, with the amino acid sequence of ACSSSPSKHCG, has been reported to have the potential ability to facilitate transdermal delivery of macromolecules such as insulin through skin. Here we report that TD1-GGS-eGFP (TGFP), or the N-terminal TD1 modified green fluorescent protein (GFP), compared to the unmodified GFP, is more capable of transporting through skin barrier in comparison with the unmodified GFP. This finding may provide a new way for transdermal drug delivery. It can also offer a new experimental methodology for the elucidation of the mechanism through which TD1 peptide facilitates transdermal delivery.

Key words: TD1; transdermal; green fluorescent protein (GFP); TD1-GGS-eGFP (TGFP)

CLC number: Q816 **Document code**: A

TD1 修饰绿色荧光蛋白透皮功能的研究

孙 铮, 温龙平

(中国科学技术大学生命科学学院, 合肥微尺度物质科学国家实验室, 安徽合肥 230027)

摘要: TD1 作为一种含有 11 个氨基酸的短肽, 具有良好的促进蛋白类大分子透皮的功能. 过去的研究显示 TD1 可以有效协助胰岛素通过皮肤进入循环并最终降低血糖. 在本研究中我们构建了一种 TD1 N 端修饰的 GFP 融合蛋白(TGFP). 我们的实验表明, 与 TD1 与 GFP 蛋白的混合物相比, TGFP 具有更加良好的透皮功能. 这一发现为透皮给药研究提供了一条新的途径, 并对解释 TD1 透皮功能具有指导意义.

关键词: TD1; 透皮; 绿色荧光蛋白(GFP); 融合蛋白(TGFP)

0 Introduction

Efficient transdermal delivery of macromolecules remains problematic because of the barrier of largely impermeable skin. Over the past decade, many related innovations in drug delivery systems have led to the development of various novel treatments with existing drugs. Several

Received: 2008-04-07; **Revised**: 2008-04-21

Foundation item: Supported by National basic research program of China, also called 973 program (2006CB933300) and The cultivation fund of key scientific and technical innovation project, ministry of education of China (706035).

Biography: Sun Zheng, male, born in 1983, master. Research field: transdermal delivery enhancer. E-mail: sunzheng@mail.ustc.edu.cn

Corresponding author: WEN Long-ping, professor. E-mail: lpwen@ustc.edu.cn

classes of surfactants, fatty acids, fatty esters and Azone-like compounds have been used as chemical penetration enhancers (CPEs) for more than 50 years. However, most of these CPEs were found to suffer from problems such as skin irritation, low efficiency in delivering large molecules and excessive dependence on physical enhancement[1~3]. The situation of CPEs research appears to be in a dilemma.

In contrast, recently several other non-chemical strategies such as the use of biological molecules have been developed and are receiving a lot of attention. The family of protein transduction domains (PTDs) was known at first for their remarkable ability to rapidly cross cell membrane and the wide range for target cells and cargos[4~6]. Most PTDs are protein motifs or sequences found in naturally occurring proteins. Among the best-known PTDs are the HIV transcription factor TAT, the Antp peptide derived from the *Drosophila melanogaster* homeodomain protein, the herpes simplex virus protein VP22, and the arginine oligomers[6,7]. In addition to their ability to transport the cell membrane in mouse tissues such as heart, lung and spleen[8], further study also reported their outstanding ability to carry compounds into animal skin[7,9~13]. The application of PTDs provides a strong potential for protein therapies such as ischemia[14,15], cancer[16,17] and some other diseases.

Conventionally a therapeutical protein and a transdermal facilitating peptide can be combined either covalently or non-covalently before being delivered. Several PTDs, such as YARA[18], TAT[9,12], Biotin-r7[19] and Antp[13], etc., have been conjugated with compounds to facilitate transdermal delivery. On the other hand, it is also reported that several arginine-rich intracellular delivery (AID) peptides not covalently attached to model proteins can promote the entry of the model protein into mouse skin in vivo.[20] Recent research by Lopes et al[18]. showed that, selected non-covalently linked PTDs can be used as a penetration enhancer, but greater skin penetration efficiency can be achieved by covalently binding the PTD to the therapeutic agent.

TD1, an innovative peptide selected by phage display, has recently been reported as an efficient non-covalent transdermal drug delivery enhancer for therapeutic proteins such as insulin[21]. To further investigate the transdermal potency of the covalently conjugated TD1 and cargo protein, we constructed TD1-eGFP (TGFP) fusion protein based on the past research that GFP is a qualified candidate to be the indicator fused with PTDs[22]. Our results indicated that TD1 associated with green fluorescent protein has significant ability to transport through skin barrier in comparison with the unmodified GFP.

1 Materials and methods

1.1 Materials

All restriction enzymes, T4 DNA ligase, dNTPs, Taq DNA polymerase and T4 polynucleotide kinase were from TaKaRa Biotech (Japan). Isopropylh-D-thiogalactoside (IPTG) was bought from Sigma (USA). The primers were synthesized by Sangon Corp (Shanghai, China). GFP antibody was from PTGLAB Corp (China), anti-rabbit IgG and His-tag antibody were from Genscript Corp (USA) and TD1 antibody was purified from anti-TD1 rabbit serum by Novomed Corp (Shanghai, China). Centriplus centrifugal filter devices (10 kD) was from Millipore (USA). BCA Protein Assay Kit was purchased from Beyotime (China). DAB kit was from Zhongshan Goldenbridge Corp (Beijing, China). EZ-ECL kit was purchased from biological industries (Israel).

1.2 Bacterial strains and plasmids

The E. coli strain Rossetta (DE3) was used as the host strain for the plasmid with E. coli GFP gene. Plasmid pET-22(+) was used to construct pET-22b (+)GFP, which overproduced the GFP and TGFP.

1.3 Plasmid constructions

The GFP clone was kept by our lab. The 5′

primer (5′-TATACATATG GCTTGTTCTTCTAGT CCTTCTAAGCATTGCGGTGGTGGCTCTATGGTGAGCAAGGGCGAG-3′) and 3′primer (5′-GAGTGCGGCCGCTCTAGATCCGGTGG ATCC-3′) were used to amplify the fusion gene TD1-GGS-eGFP. Then the gene was cloned into pET-22b(+) vector, which carries a C-terminal 6 x histidine-tag for further purification.

1.4 Purification of TGFP

All purification steps were carried out at room temperature and samples of all fractions were analyzed by SDS-PAGE. E. coli Rossetta (DE3)/pET-22b(+) TGFP or GFP was grown in 800 ml Lauria broth to A600 nm = 0.8 and then induced with IPTG (1 mmol/L) for 4 h. The cells were harvested by centrifugation, resuspended in 25 mL lysis buffer (50 mmol/L Tris, 50 mmol/L NaCl, 10 mmol/L 2-mercaptoethanol, 10% (v/v) glycerol, pH7.4). The harvested cells were disrupted with French pressure cell (Thermo Spectronic, Rochester, NY) at 20 000 psi (psi = 6.895 kPa). Cell debris was removed by centrifugation at 18 000 g for 15 min. The supernatant (25 mL) was loaded onto a 3 mL Ni^{2+}-Chelating Sepharose Fast Flow column, mixed gently, and incubated for 20 min. Then the column was washed with 60 mL of washing buffer (50 mmol/L Tris, 50 mmol/L NaCl, 10 mmol/L 2-mercaptoethanol, 20 mmol/L imidazole, 10% glycerol, pH7.4). Bound proteins were eluted with 6 mL elution buffer (50 mmol/L Tris, 50 mmol/L NaCl, 10 mmol/L 2-mercaptoethanol, 500 mmol/L imidazole, 10% glycerol, pH7.4). The imidazole was removed via centrifugal filter devices (10 kD) and the eluted protein was storaged in storage buffer (50 mmol/L Tris, 50 mmol/L NaCl). Protein concentration was measured with BCA Protein Assay Kit.

1.5 Western-blot assay for fusion proteins

5 μg GFP and TGFP were separated by 15% Tris-Tricine SDS-PAGE. Separated proteins in the gels were electrophoretically transferred onto nitrocellulose membrane at 380 mA for 45 min. The blotted membrane was blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T buffer) for 1h. After washing the membrane with PBS-T, the TD1 antibody diluted 1∶500 and GFP antibody diluted 1∶1 000 into 10 mL PBS-T were added and incubated respectively overnight at 4 ℃. After incubation, the membranes were washed with PBS-T and incubated with 1∶2 000 diluted anti-rabbit Ig secondary antibody. Then the ECL test was carried on following the manufacturer's instruction.

1.6 Spectral measurements

The fluorescence measurements were made with a Spectronic Series2 Luminescence Spectrometer (Thermo Spectronic, Rochester, NY). 5 μmol/L GFP and TGFP were excited at an EX wavelength of 488 nm. Scanning range was set from 450.0 nm to 650.0 nm. The manufacture's instructions were followed for all assay procedures.

1.7 The fluorescent microscopy of skin sections

The exposed abdominal skins of rats were treated with GFP (100 μg) and TGFP (100 μg) with or without TD1 (500 μg). The incubation time was set to 30 min. After treatment, the skin was carefully cleaned with 70% isopropyl alcohol, harvested and fixed with ice-cold 4% paraformaldehyde overnight. After washing, skin samples were immersed in 4.5% sucrose for 24 h and then dehydrated in 30% sucrose till deposition. Floating horizontal and vertical sections with a thickness of 20 μm were obtained on a freezing microtome (LEICA). We took 10 vertical sections from each skin sample and the horizontal sections were taken from 0～200 μm for every 20 μm. Fluorescence photomicrographs of the sections were obtained with OLYMPUS IX-70 microscope using a filter set having excitation and emission wavelength at 490～495 nm and 520～530 nm and ZEISS LSM-510 Confocal Laser Scanning Microscope with 488 nm excitation wavelength. All the photos were taken with exactly the same setting.

1.8 The immunohistochemical analysis of skin section

The skin sections were obtained as mentioned above and mounted on poly-L-lysine-coated slides. The sections were immersed into 0.25% Triton X-100 at 37 ℃ for 15 min and washed with PBS-T buffer (10 mmol/L PBS, 0.1% Tween200), then 3% BSA diluted in PBS-T was used to block nonspecific binding at 37 ℃ for 1 h. After incubation, anti-histag antibody diluted in 1∶200 PBS-T was added at 4 ℃ for 24 h and then washed with PBS-T buffer. The sections were then incubated with anti-rabbit Ig secondary antibody diluted 1∶500 in PBS-T at 37 ℃ for 1 h. The immunostaining with DAB kit was performed following protocols suggested by the manufacturer. After immunostaining the sections were dried at 25 ℃ and enveloped with 10 mL VECTASHIELD Mounting Medium. Fluorescence photomicrographs of the sections were obtained with OLYMPUS IX-70 microscope.

2 Results

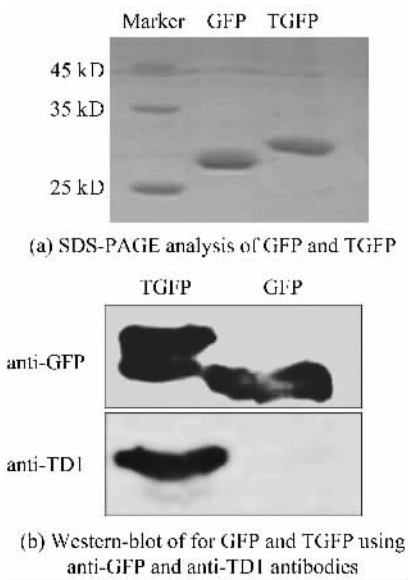
2.1 Characterization of fusion proteins

The GFP gene and TD1-GGS-eGFP gene were inserted into the multiple cloning site between the T7 promoter and T7 terminator of the expression vector of pET-22b (+) and expressed in E. coli respectively. After the induction with 1 mmol/L IPTG in 37 ℃ for 4 h, cells were harvested and inducted proteins were purified. PAGE was performed with purified proteins. As shown in Fig. 1(a), the two proteins appear to be in the size calculated from the nucleotide sequence (GFP Mr=29 371.09 Da and TGFP Mr=30 416.26 Da) respectively.

The presence of GFP and TD1 peptide domains in TGFP was further demonstrated with Western-blot with GFP antibody and TD1 antibody (Fig. 1(b)) respectively, which indicated the structure of fusion protein was as planned.

2.2 Spectral properties of TGFP

When the excitation wavelength was set to 488 nm, both purified GFP and purified TGFP emitted the same green color. Moreover, the two proteins had almost completely overlapped emission spectrums (Fig. 2), suggesting that TGFP could be useful in the transdermal assays.



(a) SDS-PAGE analysis of GFP and TGFP

(b) Western-blot of for GFP and TGFP using anti-GFP and anti-TD1 antibodies

Fig. 1　Identification of fusion proteins

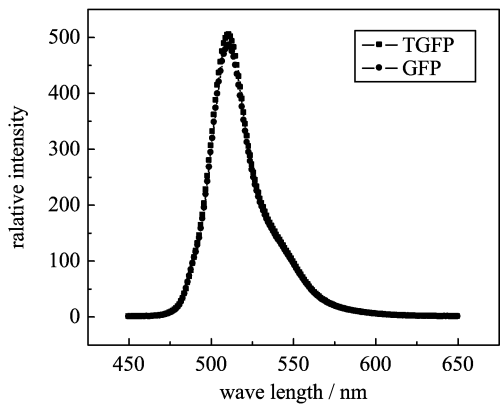
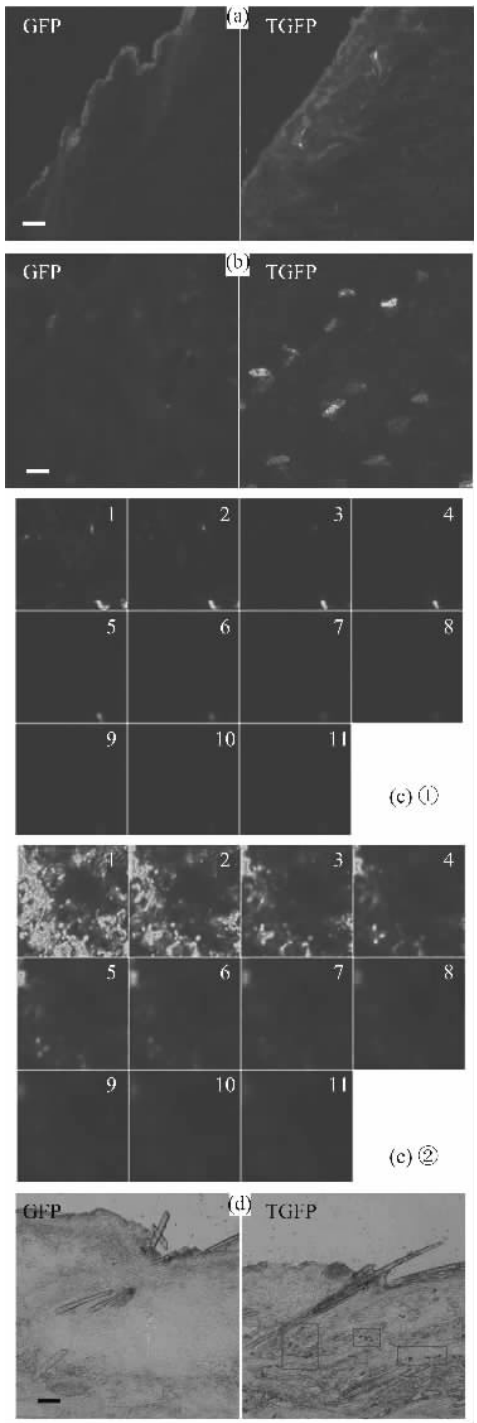


Fig. 2　Spectroscopic analyses of His-tagged E. coli GFP and TGFP emission spectrums with an exciting wavelength of 488 nm

2.3 Transdermal assay for fusion proteins

Based on that GFP and TGFP have similar spectral properties, we treated the abdominal skin of rats as mentioned above and investigated the skin sections under Fluorescent Microscopy. Fig. 3 shows that the skin sections treated with TGFP have a much stronger fluorescence than the control sections treated by GFP, which indicates that the fusion proteins may have stronger transdermal



(a) The Fluorescent Microscopy of vertical slices;
(b) The Fluorescent Microscopy of horizontal slices;
(c) Confocal Laser Scanning Microscopy of skin sections, 11 photograghs were taken automatically from 0~200 μm for every 20 μm;
① Confocal Laser Scanning Microscopy of GFP,
② Confocal Laser Scanning Microscopy of TGFP;
(d) The immunohistochemical staining Microscope of skin section 100 μg GFP and TGFP were incubated with the abdominal skin of rats respectively

Fig. 3 Transdermal effect of fusion proteins (Bar=100 μm)

ability than control proteins. We further utilized the immunohistochemical staining to confirm our fluorescence microscopic data. As shown in Fig. 3 (d), the brown staining spots were found in TGFP-treated skin but not GFP-treated skin, indicating that the fusion proteins have the transdermal ability due to the presence of TD1 peptide domain.

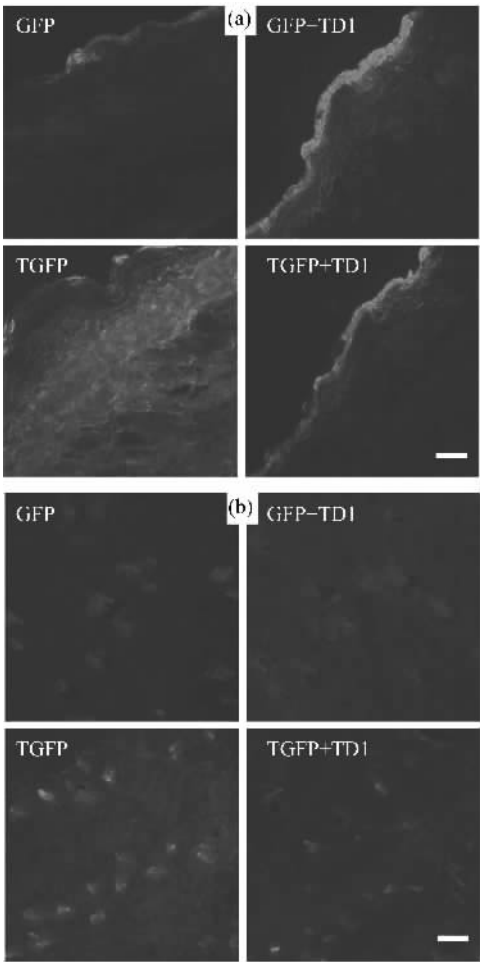
2.4 The competitive inhibiting effect of TD1 peptide to fusion proteins

In order to further investigate the transdermal mechanism of fusion proteins, we applied TD1 peptide to inhibit the transdermal ability of TGFP. As shown in Fig. 4, co-treatment of TD1 peptide with TGFP results in significant reduction of the ability of TGFP to penetrate skin while TD1 and GFP co-treatment did not significantly change the transdermal ability of GFP.

3 Discussion

Skin, as one of the most important organ of human body, is composed of ceramides, cholesterol and free fatty acids, which constitute a tight barrier to both microbial infection and therapeutic agents[19]. As mentioned earlier, many PTDs were reported to be able to facilitate proteins to penetrate animal skin both in a covalent and non-covalent manner. A recent study has shown that PTDs had a better efficiency to carry covalently conjugated compounds and penetrate the skin in vivo[18]. In our current study, we showed that TD1, like YARA and WLR, has better ability to facilitate transdermal peptide delivery when conjugated with cargo protein (GFP) covalently than non-covalently. As indicated in Fig. 3, the TGFP treated skin samples display much stronger fluorescence than the control samples treated by GFP without TD1. And the immunohistochemical staining confirms the result from another aspect. Through this study we provide a simple and useful strategy for the application and delivery of vaccines, new drugs and protein therapies.

Even though the skin penetration mediated by



(a) The Fluorescent Microscopy of vertical slices; (b) The Fluorescent Microscopy of horizontal slices in the depth of 100 μm

Fig. 4　The competitive inhibiting effect of TD1 peptide to fusion proteins (Bar=100 μm)

different PTDs has been reported in the literature[12,13,19,23], the exact mechanism remains unknown. Due to their difference in composition, the mechanism for PTDs to penetrate in the skin is likely different from that of cellular membrane penetration. Some PTDs are reported to interact with lipids[24], which facilitate them to transport across the skin. The transdermal mechanism of TD1 is also uncertain. Past research proposed that the TD1 and insulin non-covalently mixture is likely to apply the follicular penetration pathway[21]. In our research, as revealed in Fig. 4, we found that high concentration TD1 peptide has a significant competitive inhibiting effect on TGFP, which indicates that the TGFP and TD1 may share the same path way to travel through skin barrier. One of the possible reasons for TD1 to facilitate insulin delivery into the skin following a non-covalent manner but the mixture of TD1 and GFP to have little transdermal ability may be due to the difference in cargo molecular weights. Previous research demonstrated that the drug size is crucial in the delivery of therapeutic molecules mediated by CPEs[25]. Insulin (5.7 kD) is much smaller than GFP (30 kD), therefore insulin may pass through the "channel" more easily and quickly. Though GFP barely has any transdermal ability, the covalent attachment of TD1 and GFP appears to make the fusion proteins have a much better chance to be brought across the skin tissues.

4　Conclusion

In summary, we conclude that as a potential transdermal enhancer, TD1 has considerable transdermal efficiency non-covalently and maybe covalently (further investigation needed) when the cargo is small. However, when the cargo is big to a certain degree, a covalent association between TD1 and therapeutic proteins might be crucial to overcome the skin barrier. The exact mechanism underlying this effect is to be further investigated in future.

References

[1] Kalia Y N, Naik A, Garrison J, et al. Iontophoretic drug delivery[J]. Adv Drug Deliv, 2004, 56:619-658.
[2] Lavon I, Kost J. Ultrasound and transdermal drug delivery [J]. Drug Discov Today, 2004, 9:670-676.
[3] Prausnitz M R. Microneedles for transdermal drug delivery[J]. Adv Drug Deliv Rev, 2004, 56:581-587.
[4] Bogoyevitch M A, Kendrick T S, Ng D C, et al. Taking the cell by stealth or storm? Protein transduction domains (PTDs) as versatile vectors for delivery[J]. DNA Cell Biol, 2001, 21: 879-894.
[5] Hou Y W, Chan M H, Hsu H R, et al. Transdermal delivery of proteins mediated by non-covalently associated arginine-rich intracellular delivery peptides [J]. Experimental Dermatology, 2007, 16(12):999-1 006.

[6] Synder E L, Dowdy S F. Recent advances in the use of protein transduction domains for the delivery of peptides, proteins and nucleic acids in vivo[J]. Expert Opin Drug Deliv, 2005, 2: 43-51.

[7] Lopes L B, Brophy C M, Furnish E, et al. Comparative study of the skin penetration of protein transduction domains and a conjugated peptide[J]. Pharm Res, 2005, 22: 750-757.

[8] Fawell S, Seery J, Daikh Y, et al. TAT mediated delivery of heterologous proteins into cells[J]. Proc Natl Acad Sci USA, 1994, 91:664-668.

[9] Jin L H, Bahn J H, Eum W S, et al. Transduction of human catalase mediated by an HIV-1 TAT protein basic domain and arginine-rich peptides into mammalian cells[J]. Free Radic Biol Med, 2001, 31:1 509-1 519.

[10] Park J, Ryu J, Jin L H, et al. 9-Polylysine protein transduction domain: enhanced penetration efficiency of superoxide dismutase into mammalian cells and skin [J]. Mol Cells, 2002, 13: 202-208.

[11] Robbins P B, Oliver S F, Sheu S M, et al. Peptide delivery to tissues via reversibly linked protein transduction sequences[J]. BioTechniques, 2002, 33: 190-194.

[12] Lim J M, Chang M Y, Park S G, et al. Penetration enhancement in mouse skin and lipolysis in adipocytes by TAT-GKH, a new cosmetic ingredient[J]. Cosmet Sci, 2003, 54: 483-491.

[13] Schutze-Redelmeier M P M, Kong S, Bally M B, et al. Antennapedia transduction sequence promotes anti tumour immunity to epicutaneously administered CTL epitopes[J]. Vaccine, 2004, 22:1 985-1 991.

[14] Cao Guo-dong, Pei Wei, Ge Hai-liang, et al. In vivo delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis[J]. Neurosci, 2002, 22:5 423-5 431.

[15] Asoh S, Ohsawa I, Mori T, et al. Protection against ischemic brain injury by protein therapeutics[J]. Proc Natl Acad Sci USA, 2002, 99:17 107-17 112.

[16] Fulda S, Wick W, Weller M, et al. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo[J]. Nat Med, 2002, 8:808-815.

[17] Harbour J W, Worley L, Ma D, et al. Transducible peptide therapy for uveal melanoma and retinoblastoma [J]. Arch Ophthalmol, 2002, 120:1 341-1 346.

[18] Lopes L B, Furnish E, Komalavilas P, et al. Enhanced skin penetration of P20 phosphopeptide using protein transduction domains[J]. EJPB, 2008, 68: 441-445.

[19] Rothbard J B, Garlington S, Lin Q, et al. Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation[J]. Nat Med, 2000, 6(11):1 253-1 257.

[20] Wang Ya-hui, Chen Chung-pin, Chan Ming-huan, et al. Arginine-rich intracellular delivery peptides noncovalently transport protein into living cells[J]. Biochem. Biol Res Commun, 2006, 346:758-767.

[21] Chen Yong-ping, Shen Yuan-yuan, Guo Xin, et al. Transdermal protein delivery by a coadministered peptide identified via phage display [J]. Nat Biotechnol, 2006, 24(4):455-460.

[22] Chang M, Chou J C, Lee H J. Cellular internalization of fluorescent proteins via arginine-rich intracellular delivery peptide in plant cells[J]. Plant Cell Physiol, 2005, 46:482-488.

[23] Tessier D J, Komalavilas P, Liu B, et al. Transduction of peptides analogs of the small heat shock-related protein HSP20 inhibits intimal hyperplasia[J]. Vasc Surg, 2004, 40:106-114.

[24] Thoren P E, Persson D, Esbjorner E K, et al. Membrane binding and translocation of cell penetrating peptides[J]. Biochemistry, 2004, 43:3 471-3 489.

[25] Bos J D, Meinardi M M. The 500 Dalton rule for the skin penetration of chemical compounds and drugs[J]. Exp Dermatol, 2000, 9:165-169.