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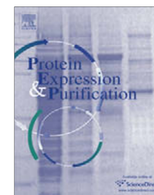

Cloning, expression, purification, distribution and kinetics characterization of the bacterial β-galactosidase fused to the cytoplasmic transduction peptide *in vitro* and *in vivo*

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A B S T R A C T

Cytoplasmic transduction peptide (CTP) offers exciting therapeutic opportunities for the treatment of many diseases caused by cytoplasmic functional molecules. It can transduce large, biologically active proteins into the cytoplasmic compartment of several mammalian cells. However, other intriguing features of CTP, including its activity *in vitro*, and distribution and tissue infiltration abilities *in vivo*, remain to be explored. The present study was initiated to (1) further confirm the cytoplasmic localization preference and the enzymatic activity of the transduced CTP-β-gal *in vitro* and (2) examine the kinetics and tissue distribution of the CTP-β-gal fusion protein in mice. A CTP-β-gal fusion protein was expressed in *Escherichia coli* and either transduced into BaF3-BCR/ABL cells or administered intravenously into female Balb/C mice at a dose of 100 μg per mouse. Its localization in BaF3-BCR/ABL cells was evaluated by immunocytochemistry and *in situ* X-gal staining, and its distribution in various tissues was analyzed both by *in situ* X-gal staining and quantitative enzymatic activity assay. β-Galactosidase enzyme activity was observed in BaF3-BCR/ABL cells and in all tissues tested, with peak activity occurring at 15 min in most tissues and at 24 h in brain. These data will not only allow rational selection of delivery schedules for therapeutic CTP, but will also aid the use of CTP fusion protein transduction in the development of protein therapeutics targeting the cytoplasmic compartment both *in vitro* and *in vivo*.

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Introduction

The emergence of macromolecules, such as proteins and nucleic acids, as therapeutic agents has sparked a renewed interest in novel drug carriers as a means to enable these macromolecules to reach their subcellular targets. However, efficient intra-cellular delivery and correct localization of pharmaceutical macromolecules remain problematic due to the barrier of impermeable cell membranes and the absence of directing signals that are required for the specific targeting of different cell organelles. A novel approach to deliver macromolecules to intra-cellular compartments involves their attachment to peptides that transport them through cellular membranes. The protein transduction domain (PTD)[2] of HIV-1 TAT has been extensively documented with regard to its membrane transduction potential [1–3], as well as its efficient delivery and tissue distribution of biomolecules *in vivo* [4–6], and the detailed kinetics and tissue distribution of its fusion protein in mice [7]. However, due to its functional nuclear localization sequence (NLS), the majority of PTD and PTD-conjugated molecules translocate to the nucleus rather than to the cytoplasm after transduction; hence, they are unsuitable for cyto-

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[2] *Abbreviations used:* PTD, protein transduction domain; NLS, nuclear localization sequence; CTP, cytoplasmic transduction peptide; CML, chronic myeloid leukemia; BBB, blood brain barrier; PBS, phosphate buffered saline; ECL, enhanced chemiluminescence.

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plasmic targeting. Cytoplasmic transduction peptide (CTP) is a newly designed transduction peptide that carries molecules across the cell membrane with a preference to localize to the cytoplasmic compartment [8]. Therefore, as a biological tool, CTP provides therapeutic potential for many diseases caused by malfunctioning cytoplasmic molecules. Indeed, since its introduction into the biomedical field, data from others [9–11] and our own group [12] have further demonstrated that CTP-fused proteins exhibited a clear preference for cytoplasmic localization, markedly enhanced membrane transduction potential and remarkable remedial effects *in vitro*. Furthermore, in an *in vivo* trafficking study conducted by Kim et al. [8], CTP-fused β-galactosidase (CTP-β-gal) exhibited unique organ tropisms to the liver and lymph nodes when systemically injected into mice. However, other intriguing features of CTP, including its activity *in vitro* and its distribution and tissue infiltration abilities *in vivo*, require further elucidation.

Homodimerization of the cytoplasmic BCR–ABL oncoprotein mediated by its N-terminal oligomerizaton (OD) domain plays a key role in the pathogenesis of chronic myeloid leukemia (CML), and our previous study [12] has demonstrated efficient interruption of BCR–ABL homodimerization by CTP-mediated delivery of OD in the form of a CTP–OD–HA fusion peptide (HA tag was introduced to simplify immunological detection) in K562 cells. Subsequent evaluation of its therapeutic effect on the *in vivo* CML animal model is urgently needed. However, up till now, there is still little information on the *in vivo* model of CTP fusion proteins and the evaluation of the detailed kinetics and tissue distribution of CTP fusion proteins becomes a prerequisite for our further evaluation of the therapeutic effect of the CTP–OD–HA peptide on the CML animal model. In particular, further demonstration of the potential ability of the CTP fusion peptide to penetrate the blood brain barrier (BBB) is urgently needed for further evaluation of the therapeutic effect of the CTP–OD–HA fusion peptide on the treatment of the minimal residual leukemia found in the brain. In addition, further information is required regarding the CTP fusion protein and its viability in human studies. Thus, the current experiments were designed to (1) confirm the cytoplasmic localization preference and the enzymatic activity of the transduced CTP-β-gal fusion protein in the murine BaF3-BCR/ABL cells; (2) examine the kinetics and tissue distribution of the CTP-β-gal fusion protein in mice to better understand its deposition in humans.

To achieve this goal, the prokaryotic expression vector for the CTP-β-gal fusion protein was constructed and the protein was purified after its expression in *Escherichia coli* BL21 (DE3) bacteria. The membrane transduction potential, cytoplasmic localization preference and potent enzymatic activity of the CTP-β-gal fusion protein were first evaluated in cultured BaF3-BCR/ABL cells *in vitro*, which were established by insertion and expression of human bcr–abl cDNA in the murine pre-B lymphocytic cell line BaF3. Furthermore, its detailed kinetics and tissue distribution in Balc/C mice were evaluated after intravenous (i.v.) administration. BaF3-BCR/ABL cells and Balc/C mice were selected because Balc/C mice infused with BaF3-BCR/ABL cells exhibited rapid infiltration of lymphoblastic BaF3-BCR/ABL cells into a variety of tissues. This resulted in a syndrome resembling the blast-crisis phase of CML or acute lymphoid leukemia, thus facilitating our further evaluation of CTP–OD–HA therapeutic peptides [12] in leukemia animal models. Our data demonstrated that CTP-β-gal was remarkable in terms of its cytoplasmic localization ability, and also exhibited potent membrane transduction potential into the BaF3-BCR/ABL cells. In the *in vivo* studies, CTP-β-gal was found to be more efficient than PTD-β-gal in its ability to infiltrate various tissues as evidenced by the potent enzymatic activity in all the tissues tested compared with the previously reported activity for PTD-β-gal [7].

Materials and methods

Animal experiments

These studies were conducted in accordance with the animal care guidelines instituted by the Animal Studies Committee of Chongqing Medical University.

Twenty-eight 4- to 6-week-old Balc/C female mice (Central Laboratory of animal facility, Chongqing Medical University, Chongqing, China) were maintained at the Animal Facility of Chongqing Medical University. Animals were kept in filter-top cages on sterile bedding and provided with sterile food and acidic water *ad libitum*. Mice were injected with 100 μg CTP-β-gal protein (equivalent to 5 mg/kg body weight), i.v. via the tail veins. Negative control mice received the same volume of phosphate buffered saline (PBS) and the experimental control mice were injected with the same dose of β-galactosidase protein. Tissues were harvested 15 min, 1 h, 3 h and 24 h after treatment. Half of each tissue was collected for whole mount and cryo-section slide-based enzyme histochemistry of 5-bromo-4-chloro-3-indolyl-β-D-galatopyranoside (X-gal) staining at necropsy, and the other half was placed into liquid nitrogen for the quantitative β-galactosidase enzyme activity assay.

pCTP-lacZ cloning and expression

The T7-RNA polymerase based pET-32a(+) vector, in which the genes of interest could be cloned downstream to the *E. coli* thioredoxin (TRX) in order to increase the solubility of the target protein, was employed. Two stages were involved in the construction of the bacterial expression plasmid for CTP-β-gal. First, a cDNA sequence encoding the whole open reading frame of the *E. coli*-derived β-galactosidase protein was generated by a PCR strategy from the pCMVβ-lacZ plasmid containing the complete CDS for bacterial β-galactosidase (GenBank Accession No. J01636). A 5′ primer containing the Bam HI and a 3′ primer containing the Hind III site were used to generate a 3162 bp PCR product from Bam HI to Hind III. Sequences of the paired primers used for PCR amplification were presented as follows: lacZ-F, 5′-CGCGGATCCAGCATGTCGTTTACTTT-3′ and lacZ-R, 5′-CCCAAGCTTCCCTTATTTTTGACACCAG-3′. Underlined nucleotides represent the Bam HI and Hind III sites, respectively. After being subcloned and completely sequenced to verify the correct reading frame and absence of mutations, the lacZ cDNA was introduced as a Bam HI to Hind III fragment into the unique Bam HI to Hind III sites of the prokaryotic expression vector, pET-32a(+). From this, the bacterial β-galactosidase protein can be expressed as a chimera with *E. coli* thioredoxin (TRX), thus acquiring the pET32a-lacZ plasmid, which was further identified by restriction enzyme digestions and bacterial colony PCR. Second, the 41 nt antisense (AS) and sense (S) oligonucleotides encoding the CTP peptide containing the NcoI and BamHI sites and phosphorylated at their 5′ end were synthesized, self-annealed and inserted into the NcoI and BamHI sites of the pET32a-lacZ plasmid, finally producing the pCTP-lacZ vector. Sequences of the inserts were: CTP-S, 5′-pCATGGATTACGGACGCCGCGCACGCCGCCGCCGCCGCCGCG-3′; CTP-AS, 5′-pGATCCGCGGCGGCGGCGGCGGCGTGCG CGGCGTCCGTAATC-3′. Underlined nucleotides represent the NcoI and BamHI sites, respectively. Furthermore, the pCTP-lacZ plasmid was identified by restriction enzyme digestions, colony PCR and bidirectional DNA sequencing, and the clones with an insert orientation preserving the direction of transcription from the T7lac promoter were selected. The CTP-lacZ expressing bacteria were subsequently obtained by transient transformation of the constructed pCTP-lacZ recombinant plasmid into the BL21 (DE3) expression strain by calcium chloride and the positive expression clones were subsequently screened out by colony PCR.

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