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# Cloning, expression, purification and functional characterization of the oligomerization domain of Bcr–Abl oncoprotein fused to the cytoplasmic transduction peptide

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ABSTRACT

Protein-based cellular therapeutics have been limited by getting molecules into cells and the fact that many proteins require accurate cellular localization for function. Cytoplasmic transduction peptide (CTP) is a newly designed transduction peptide that carries molecules across the cell membrane with a preference to localize in the cytoplasmic compartment and is, therefore, applicable for cytoplasmic targeting. The Bcr–Abl fusion protein, playing major causative role in chronic myeloid leukemia (CML), is a cytoplasmic oncoprotein that contains an N-terminus oligomerization domain (OD) mediating homodimerization of Bcr–Abl proteins, and an intact OD in Bcr–Abl is required both for the activation of its transforming activity and tyrosine kinase. Therefore, disrupting Bcr–Abl oligomerization represents a potential therapeutic strategy for inhibiting Bcr–Abl oncogenicity. In this study, we explored the possible homodimerization-disrupting and tyrosine kinase inhibiting effect of the transduction of OD in Bcr–Abl positive K562 cells. By expressing in *Escherichia coli* a CTP-OD-HA fusion protein followed by $Ni^{+}$–NTA affinity purification, immunoblot identification and enterokinase cleavage, we showed that the CTP-OD-HA protein was structurally and functionally active in that it potently transduced and primarily localized into the cytoplasmic compartment, heterodimerized with Bcr–Abl, and potently inhibited the phospho-tyrosine pathways of Bcr–Abl oncoprotein at a low concentration of 4 μM. These results delineate strategies for the expression and purification of therapeutic molecules for intracytoplasmic protein based therapeutics and the CTP-OD-HA-mediated killing strategy could be explored as a promising anti-leukemia agent or an adjuvant to the conventional therapeutic modalities in chronic myeloid leukemia, such as in vitro purging.



## Background

The Bcr–Abl oncoprotein is responsible for a wide range of human leukemias including most cases of Philadelphia chromosome-positive chronic myeloid leukemia (CML). The deregulated Abl tyrosine kinase of Bcr–Abl fusion protein plays a major causative role in the pathogenesis of that hematopoietic malignancy and a better understanding of the regulation of Bcr–Abl kinase activity is essential for rational drug development for CML. Recently [1], a simple crystal model suggested that homodimerization of the Bcr–Abl fusion protein through the N-terminal coiled-coil domain of the Bcr-portion might constitutively dysregulate the Abl kinase of the Bcr–Abl oncoprotein. Moreover, using novel Bcr coiled-coil mutants, Smith et al. [2] have provided direct evidence that oligomerization of Bcr–Abl is the important kinase-activating function of the coiled-coil domain. The Bcr–Abl coiled-coil deletion and alanine substitution mutants failed to oligomerize, were defective for transformation of fibroblasts and primary B-lymphoid cells, and were unable to induce CML-like myeloproliferative disease in mice. Because both the tyrosine kinase and oligomerization domain are essential for Bcr–Abl oncogenicity, they may independently serve as drug targets. A specific kinase inhibitor has been shown to target the Abl kinase domain and has been clinically effective in the treatment of CML [3]. However, drug-resistant leukemia cells have been

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observed in CML patients following drug treatment [4]. Targeting the Bcr oligomerization domain may provide a complementary therapeutic approach to specifically disrupt Bcr–Abl oncogenic potential. Actually, the enforced expression of a peptide including the Bcr–Abl oligomerization domain from the plasmid vector containing the target gene has previously been shown to inhibit Bcr–Abl oligomerization in a dominant-negative manner and to further potently reduce the transformation efficiency of the Bcr–Abl expressing cell lines [5]. Nonetheless, due to the large doses required to achieve therapeutic expression levels of intended proteins and the potential integration of the vector genes with the host chromatin material, safety remains a serious concern for the ultimate clinical application of various gene-expressing vectors [6–9]. An alternative approach that appears to be the safest is to produce recombinant proteins exogenously and then deliver them systemically or by localized injections into the target organs. Discovery of the HIV Tat protein transduction domain (PTD) has opened avenues for directing in vitro and in vivo delivery of proteins into cells. However, due to the functional nuclear localization sequence (NLS) in PTD, the majority of PTD and PTD-conjugated molecules translocate to the nucleus rather than to the cytoplasm after transduction [10,11]. Actually, results from many studies have strongly suggested that the cytoplasmic proteins or other biomolecules, targeting cytoplasmic compartments could be localized into the nucleus after membrane transduction when they were conjugated with PTD, which may induce unexpected responses or untoward effects on the target cells [11–15]. Recently [11], a cytoplasmic transduction peptide (CTP),[2] which was deliberately designed to ensure the efficient cytoplasmic delivery of the CTP-fused biomolecules has been reported. In comparison with PTD, CTP and its fusion partners have been shown to exhibit a clear preference for cytoplasmic localization and a markedly enhanced membrane transduction potential, implying that CTP can be taken as a novel delivery peptide appropriate for molecular targeting to cytoplasmic compartments in vitro without causing any untoward effects on nuclear genetic materials. Therefore, CTP is predicted to be useful in the delivery of biomolecules into cytoplasm, especially of those compounds which are specific to cytoplasmic targets but exhibit poor bioavailability, and this ability of CTP may prove quite useful in the development of new treatments for a host of diseases.

In the present study, The T7-RNA polymerase based pET system was employed to express the mammalian protein in *Escherichia coli* (*E. coli*). OD, HA and CTP fragments were subsequently cloned into pET-32a(+) vector, in which the genes of interest were cloned downstream to the *E. coli* thioredoxin (TRX) chimera in order to increase the solubility of the target protein and tagged with an N-terminal His tag to allow easy purification. Furthermore, we have examined the ability of the purified CTP-OD-HA fusion protein to penetrate into the cell membrane, to localize in the cytoplasmic compartment, to heterodimerize with Bcr–Abl and to inhibit the tyrosine kinase of Bcr–Abl oncoprotien.

## Materials and methods

### *Bacterial strains and culture conditions*

DH5α and BL21 were used as host strains for cloning and expression experiments in *E. coli*. The *E. coli* expression vector pET-32a (+) (Novagen) was used and the transformed strains were grown in Luria–Bertani (LB) broth or on LB agar (Hi-Media) supplemented with ampicillin (100 μg/ml). All strains were incubated at 37 °C with constant shaking (220 rpm).

### *Construction of CTP-OD-HA-expressing prokaryotic vector pCTP-OD-HA*

Three stages were involved in the construction of the bacterial expression plasmid for CTP-OD-HA (see outline in Fig. 1). Firstly, a cDNA sequence encoding the whole open reading frame of the oligomerization domain (OD) of Bcr–Abl including the 1–72 aa was generated by a PCR strategy taking the Mig210 plasmid containing the complete CDS for Bcr–Abl (GenBank Accession No. for the complete CDS of bcr gene is NM_021574) as a template. A 5′ primer containing BamHI and a 3′ primer containing EcoRI site were used to generate a 237 bp PCR product from BamHI to EcoRI. After being subcloned and completely sequenced to verify the correct reading frame and lack of mutations, the OD cDNA was then introduced as an BamHI to EcoRI fragment into the unique BamHI to EcoRI sites of the prokaryotic expressing vector, pET-32a(+), from which $Bcr_{1-72}$ can be expressed as a chimera with *E. coli* thioredoxin (TRX), thus acquiring the pET32a-OD plasmid, which was further identified by restriction enzyme digestions and colony PCR. Secondly, the 36-nt antisense (AS) and sense (S) oligonucleotides, respectively, containing the EcoRI and HindIII site and phosphorylated at their 5′ end encoding the HA-tag were synthesized, self-annealed and inserted into the EcoRI and HindIII sites of the pET32a-OD plasmid, acquiring the pOD-HA recombinant prokaryotic expressing vector, which was further identified by restriction enzyme digestions and colony PCR. Sequences of the inserts were: HA-S, 5′-pAATTCTACCC ATACGACGTCCCAGACTACGCTTAGA-3′; HA-AS, 5′-pAGCTTCTAAGCG TAGTCTGGGACGTCGTATGGGTAG-3′. Underlined nucleotides represent, respectively, the EcoRI and HindIII site. Meanwhile, the 41-nt antisense (AS) and sense (S) oligonucleotides encoding the CTP peptide, respectively, containing the NcoI and BamHI site and phosphorylated at their 5′ end were synthesized, self-annealed and inserted into the NcoI and BamHI sites of the pOD-HA plasmid, finally getting the pCTP-OD-HA plasmid. Sequences of the inserts were: CTP-S, 5′-pCATGGATTACGGACGCCGCGCACGCCGCCGCC GCCGCCGCG-3′; CTP-AS, 5′-pGATCCGCGGCGGCGGCGGCGGCGTGC GCGGCGTCCGTAATC-3'. Underlined nucleotides represent the NcoI and BamHI site, respectively. Furthermore, the pCTP-OD-HA 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. All the paired primers used for PCR amplification or self-annealing were listed in Table 1. The CTP-OD-HA expressing bacteria were subsequently obtained by transient transformation of the constructed pCTP-OD-HA recombinant plasmid into the BL21(DE3) expression strain by calcium chloride and the positive expression clones were subsequently screened out by colony PCR.

### *Transformation of protease deficient BL21 cells*

Transformation of BL21 cells was carried out using heat shock at 42 °C for 1 min and chilling on ice for 2 min. A volume of 200−500 μl of LB media was added to the cells and mixed gently. The cells were then incubated at 37 °C for 1 h at 200−250 rpm in a platform shaking incubator (Bioline Series 4600, Edwards Instrument Company, Australia) before an aliquot of 100 μL was spread onto an LB/amp plate and incubated at 37 °C for 15−20 h.

### *Protein expression*

Both pCTP-OD-HA and the control pOD-HA recombinant plasmids were, respectively, transiently transformed into the *E. coli*.

[2] *Abbreviations used:* CTP, cytoplasmic transduction peptide; CML, chronic myeloid leukemia; OD, oligomerization domain; PTD, protein transduction domain; NLS, nuclear localization sequence; CTP, cytoplasmic transduction peptide; TRX, thioredoxin.