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Chimeric p53 as an alternative therapy for hypoxic tumors

Jun-Ho Lee and Hua Lu*
Department of Biochemistry and Molecular Biology; IU-Simon Cancer Center; Indiana University School of Medicine; Indianapolis, IN USA

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*Correspondence to: Hua Lu;
Email: hualu@iupui.edu

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Aside from surgical and radiological therapeutic interventions of human cancers, a number of approaches facilitating chemo- or gene therapy have been proposed and tested for their therapeutic efficacy. Examples of those approaches include delivering potential therapeutic compounds or oligonucleotides into living cells, such as via receptor-mediated endocytosis, lipid-based delivery and adeno- or retroviral delivery systems. However, in many cases, these delivery methods are often limited by small molecule size, cell type restriction, targeting inefficiency, low intracellular concentration and other biochemical properties such as long-term stability, cytotoxicity and cell permeability.[1,2] Delivery of bioactive peptides or proteins can be achieved across the cell membrane as well as the blood-brain barrier only when these molecules are small (less than 600 daltons).[3] To circumvent some of these problems, an alternative approach called 'protein transduction' or 'protein therapy' has been developed.[3] A small peptide, identified as a cell-penetrating peptide (CPP) or a protein transduction domain (PTD) peptide, is fused to a partner protein and the fusion protein is able to cross the cell membrane. Examples of PTDs include Antenapedia (Antp) from Drosophila,[4] HSV VP22 from herpes simplex virus (HSV),[5] and TAT peptide from human immunodeficiency virus (HIV).[6,7] Among these, the PTD of HIV-TAT has been in widespread use for the transduction of a variety of proteins of interest, including proteins with high molecular weights. Several studies have demonstrated that TAT-fusion proteins can be transduced easily and quickly (less than 5 min) into almost all types of cells tested in vitro.[3,8] Even though discrepancies exist,[9] amazingly, this fusion protein was also able to cross the blood-brain barrier in vivo.[3,8,10]

The protein transduction approach was first introduced in 1988 based on the observation that the full-length (86 amino acid) TAT protein of HIV is rapidly taken up by cells and transactivates the viral genome.[6,7,11] This characteristic of the TAT peptide was applied for TAT-fused heterologous proteins such as β-galactosidase, horseradish peroxidase, RNase A and domain III of Pseudomonas exotoxin A.[9] Those TAT chimeras were also effectively delivered to all the cell types tested, and in particular, a TAT-β-galactosidase fusion protein was delivered into several mouse tissues allowing the possible therapeutic delivery of macromolecules, which were previously considered to be impermeable to living cells.[9]

In 1999, Dowdy and his colleagues re-investigated the previous observation with more details by utilizing an N-terminal, 11 amino acid protein transduction domain (PTD) of HIV-TAT (TAT_{47-57}: YGRKKRRQRRR) fused to β-galactosidase.[3] Intraperitoneal injection of this biologically active macromolecule showed direct distribution to all mouse tissues including the brain, with enhanced transduction potential as well as strong and uniform β-Gal activity.[3] The applications of full-length or protein transduction domain (PTD) of TAT have been tested in various fusion partner proteins such as TAT-$p27^{Kip1}$, HIV protease-activated TAT-caspase 3, TAT-GFP, TAT-cdk2, TAT-catalase and TAT-Cu, Zn superoxide dismutase (TAT-SOD) validating direct and fast introduction of TAT-fusion proteins into cells and/or tissues in a time- and dose-dependent

manner with functional effectiveness.[12-16] Together, these studies made the protein therapy more promising for the efficient transduction of potential therapeutic proteins to patients.

Tumor suppressors exert their negative effects on tumors mainly by induction of apoptosis and DNA damage repair as well as inhibition of cell division and metastasis.[17] Among the well-studied tumor suppressors, p53 is one of the most important and adopts multiple mechanisms to kill cancer cells. The tumor suppression function of p53 is well manifested by the fact that more than half of human tumors harbor mutations in *Tp53* and the remaining tumors retain impaired functions in the p53 pathway even though they harbor wild type p53.[18-20] Therefore, restoration of wild type p53 function has been a major strategy for the development of cancer therapy. The transduction approach of TAT-fusion proteins has also been applied for delivering wild-type p53 as an anticancer therapy. The transduction ability and biological activity of the p53 fusion proteins, in which p53 is conjugated to the PTD of HIV-TAT or an even shorter N-terminal basic domain (RKKRRQ RRR) of TAT, have been tested in various cancer cells.[21,22] The TAT-p53 protein was efficiently introduced into different types of cancer cells, and could inhibit cell growth and induce apoptosis in Saos-2, HeLa, Hep G2 (wild-type p53), Hep 3B (null p53), LNCap (wild-type p53) and PC3 (null p53) cells.[21,22] These studies suggest that the introduction of cell permeable TAT-p53 fusion proteins can be an alternative anticancer treatment in various types of cancers, regardless of their p53 status.

The presence of hypoxic cells in solid tumors, but not in normal cells, has often been a major obstacle for radio- and chemotherapy.[23] However, this unique feature in solid tumors including non-small cell lung carcinoma (NSCLC) also provides an important target for selective cancer therapy. The p53 protein can be stabilized and activated in response to various stressors, including hypoxic stress.[24] Another well-studied and hypoxia-activated transcription factor is hypoxia-inducible factor (HIF). It is a heterodimeric transcription factor composed of α and β (belonging to the helix-loop-helix PAS family) and its activity is primarily regulated by hypoxia-induced stabilization of HIF-1α, which is otherwise rapidly degraded under a normoxic condition.[25,26] The responsible domain for this oxygen-dependent degradation localizes in the central region of HIF-1α[27] with a minimum oxygen-dependent domain (ODD) ($ODD_{557-574}$), which was also tested for TAT fusion proteins, such as TAT-ODD-caspase 3.[28] Studies utilizing this minimum ODD of HIF-1α have shown selective expression and stability of a fusion partner protein under hypoxic conditions.[28,29] Hence, the above pioneering studies provide a framework for the following work to test the corresponding chimeric p53 protein.

A report in the current issue of *Cancer Biology & Therapy* presented by Yu Zhao et al. demonstrates the effects of a hypoxia-targeted p53 fusion protein in NSCLC. The notion and approach for this study are a continuation of their previous observations.[30] The authors first introduced the PTD of HIV-TAT (TAT_{47-57}) fused to the minimum oxygen-dependent degradation domain ($ODD_{557-574}$) of HIF-1α, named TAT-ODD-p53. The TAT-ODD-p53 protein was tested to determine its effects on tumor growth and apoptosis in H1299 cells compared to other controls including a TAT-p53 protein. To examine the cell permeability of this fusion protein, H1299 cells were incubated along with PBS or p53, TAT-p53 and TAT-ODD-p53 proteins and analyzed by immunofluorescence. TAT-p53 treated cells showed much stronger staining intensity than TAT-ODD-p53 treated ones under normoxia (20% O_2). However, under a hypoxic condition (1% O_2), TAT-ODD-p53 staining was as strong as TAT-p53 staining, indicating that both of the chimeric proteins penetrate the cell membrane efficiently and the TAT-ODD-p53 protein is selectively degraded in normoxia via an ODD-regulated pathway, but protected under hypoxia. The staining of PBS- or p53 protein treated cells was undetectable, confirming the TAT domain is required for efficient protein transduction. The results were also confirmed by a significant extension of the half-life of TAT-ODD-p53 under hypoxia to a half-life close to that of TAT-p53. Therefore, the inhibitory effect of TAT-ODD-p53 on cell growth and viability was hypoxia-dependent, whereas that of TAT-p53 was hypoxia-independent. This appeared to be due to p21-induced G_1 arrest since more G_1 cells accumulated under hypoxia in comparison with p21 levels under normoxia. In addition, TAT-ODD-p53 induced apoptosis markedly under hypoxia in a time-dependent manner with a concomitant increase of activated caspase-3 activity. By contrast, apoptosis induced by TAT-p53 was independent of oxygen condition. Consistent with these cell-based assays, they also showed that TAT-ODD-p53 could be specifically co-distributed with HIF-1α in hypoxic tumor tissues derived from H1299 cells in immuno-deficient Balb/c mice, while TAT-p53 was widely distributed even in normal tissues. The tumor mass and volume were also reduced in TAT-ODD-p53 treated mice with no obvious side effects. Notably, different from in vitro data, TAT-p53 did not show inhibition of tumor growth and even showed slight promotion of the progression of tumorigenesis. The authors speculated that this might be due to neutralization of TAT-p53, thus resulting in a wide distribution of TAT-p53 in vivo. Alternatively, the antitumor activity might be partially derived from TAT-ODD, not only from p53. However, the latter possibility could be eliminated by another control experiment utilizing TAT-ODD-EGFP. The expression of several p53-associated genes such as p21, PUMA and caspase-3 was significantly elevated in the tumor tissues of TAT-ODD-p53 treated mice, suggesting the antitumor effect of TAT-ODD-p53 should stem from the growth arrest and apoptosis of hypoxic tumor cells via a p53-dependent pathway.

This study presents a meaningful cue for the treatment of hypoxic tumors, which are predisposed with more aggressive phenotypes and highly resistant to radio- and chemotherapy.[23] Clearance of hypoxic cells in solid tumors is essential for an effective therapy prior to the occurence of angiogenesis, and otherwise, recurrence of these malignant tumors would be followed with cell expansion, progression, invasion and metastasis. Among the various approaches to target

hypoxic tumors, the strategy to employ this chimeric p53, TAT-ODD-p53, as described in this study, is apparently a clever one, as it could be specifically activated under hypoxic conditions to kill hypoxic cancer cells by inducing apoptosis and cell growth arrest. This strategy may be particularly useful for cancer cells that are deficient in wild type p53. However, it remains unclear if this chimeric p53 would be able to overcome the dominant negative effect of some cancer-derived mutant p53 molecules in hypoxic tumor tissues derived from human cancers that harbor mutant p53. It is surprising that TAT-p53, which was expressed in almost all mouse tissues including xenograft NSCLC tumor tissues, did not show apparent inhibition of cancer cell growth in vivo. Based on the in vitro results and studies by others,[21,22] could a synergistic effect of TAT-ODD-p53 with p53-TAT be achieved to obtain more prominent and selective antitumor activity? This possibility could be eliminated because TAT-p53 might be generally toxic to normal tissues since it was expressed nonspecifically in normal tissues. Instead, a combination of TAT-ODD-p53 with other TAT-ODD fusion proteins, which contain tumor suppressive activities, might be expected to exert a certain degree of synergism.

Prior to applying the TAT-ODD-p53 chimera to clinical trials for patients with NSCLC or other solid tumors, minimally, the following questions need to be addressed. First, although TAT-fusion proteins can penetrate most cell types, if not all, rapidly in a concentration dependent fashion and appears to be independent of receptors and transporters,[3,31] the precise underlying mechanism is still elusive. Thus, elucidation of the mechanism would be not only conducive to modifying and improving TAT-fusion tactics, but also helpful for eradicating unnecessary side effects. Furthermore, to confirm the functional effectiveness and target specificity of TAT-ODD-p53 in hypoxic tumors, precise analysis with more delicate and contamination-free sample preparation by utilizing a laser microdissection for hypoxic tumor regions would firmly validate the current observations in this study. Moreover, the immune response and toxicity by a long-term treatment of this chimeric p53 protein also need to be closely investigated and systematically monitored since the present study was carried out using immuno-deficient mice. Finally, although the transduction of TAT-ODD-p53 was rapid, direct, and efficient with functional integrity of p53 specific to hypoxic NSCLC tumors under a hypoxic condition as reported in this study, future studies will also be expected to confirm these observations and validate the anticancer activity of TAT-ODD-p53 in multiple cancer cell types regardless of the status of *TP53*.

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