Protein Transduction Domain-Mediated Delivery of Anticancer Proteins

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1 Introduction

Advances in molecular and cellular biological techniques and genomic information obtained through the human genome project have been accelerating the elucidation of the molecular mechanisms underlying cancer. Both genetic mutations and epigenetic alterations have been associated with cancer [1]. The former include deletions, point mutations, or amplification of genes, chromosomal translocations, and gain or loss of entire chromosomes. The latter are modifications of genomic DNA, such as methylation and acetylation. All of these alterations lead to a gain of function of oncogenes or loss of function of tumor suppressor genes and have been recognized as effective targets for cancer therapy. Not only small chemicals but also various high-molecular weight biomacromolecules, such as oligonucleotides, antisense nucleotides, antisense peptide nucleic acids, small interference RNA, DNA (cDNA), peptides, proteins, and antibodies, have proven useful for regulating the function of these target genes. However, the plasma membrane of the cell surface forms an effective barrier and limits the internalization of such macromolecules into cells; therefore, the application of these information-rich macromolecules to cancer therapy has long been restricted. Although various methods to internalize macromolecules into living cells in vivo have been proposed, most of them resulted in inefficient delivery. Additionally, other problems such as complex manipulation, toxicity, and immunogenicity have prevented the routine therapeutic use of macromolecules.

Over the past decade, the unique activity of oligopeptides, known as protein transduction domains (PTDs) or cell penetrating peptides (CPPs), has made it possible to transduce biologically active macromolecules into living cells [2, 3]. It was accomplished by conjugating a PTD to the desired macromolecule. Various kinds of macromolecules have been successfully internalized into living

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cells and confirmed to show the expected activity in the cells. Moreover, several groups have already applied this strategy in vivo and confirmed anticancer activity in preclinical experiments using tumor-bearing animals [4–17].

In this chapter, we focus on recent progress in PTD-mediated anticancer strategies. In addition, we review the characteristics of PTD polypeptides, mechanism of PTD-mediated internalization, problems and perspectives of PTD-mediated anticancer strategies, and history of this research field.

2 PTD as a Transducer of Macromolecules into Living Cells

In 1988, two groups, Green and Loewenstein, and Frankel and Pabo, independently reported that the transcriptional activator of transcription (Tat) protein of human immunodeficiency virus-1 (HIV-1) has the unique potential to enter cultured cells (Table 1) [18, 19]. Green and Loewenstein found that a chemically synthesized partial Tat protein (first 86 amino acids of the protein) entered HeLa cells when added to the culture medium and transactivated the expression of a Tat-responsive reporter gene pretransfected in the cells. Frankel and Pabo also demonstrated that the addition of a recombinant Tat protein to the culture medium was sufficient to induce the expression of a HIV-1 LTR-dependent reporter gene which was pretransfected in HeLa cells. Although the physiological importance of this internalization still remains to be elucidated, the reports marked an important first milestone in the development of a PTD-mediated anticancer strategy.

Table 1 Milestones for the development of PTD-mediated delivery of bioactive protein in vivo

Year	Finding	References
1988	Internalization of chemically synthesized partial Tat protein (first 86 a.a.) into living cells and its bioactivity	18
	Internalization of recombinant Tat protein into living cells and its bioactivity	19
1994	Tat-mediated internalization of heterologous protein into living cells	20
	Identification of the “tat protein transduction domain”	
1999	Tat-mediated delivery of biologically active protein in vivo	39

In 1994, Fawell et al. marked a second milestone, that the Tat protein can mediate the internalization of a heterologous protein into cells by chemical conjugation (Table 1) [20]. They chemically cross-linked Tat peptides (residues 1–72 or 37–72) to β-galactosidase, horseradish peroxidase, RNase A, and domain III of pseudomonas exotoxin A (PE) and monitored their uptake. Interestingly, all the cells in the culture dish were transduced with the Tat protein. In addition, the internalization was achieved in all cell types tested, such as HeLa, COS-1, CHO, H9, NIH3T3, primary human keratinocytes, and

umbilical endothelial cells. The domain responsible for this translocation was identified in the short basic region (47–57 of the Tat protein) and termed the "Tat protein transduction domain (PTD)" [21–23]. Subsequent studies have further demonstrated that Tat-PTD facilitates the internalization of conjugated proteins into living cells in vitro [24]. Likewise, a number of other cationic peptides, e.g., a peptide from the third α–helix of the antennapedia homeodomain [25–28], and a peptide from the VP22 protein from the herpes simplex virus [29] have been reported as PTDs showing the same attractive activity as Tat-PTD [30]. Using these PTDs, various physiologically and therapeutically active macromolecules, such as peptides, proteins [20], antisense peptide nucleic acid [31, 32], DNA [33], super magnet beads [34], liposomes [35], λ phages [36], and antibodies [37] have been successfully transduced into living cells. The intracellular delivery of these macromolecules modulates the functions of various genes [30] related to the cell cycle [22] and apoptosis [38] in vitro. Moreover, in 1999, Schwarze et al. demonstrated that the intraperitoneal injection of a Tat-PTD-fused 120 kDa β-galactosidase (β-Gal) protein resulted in the delivery of the biologically active fusion protein to all tissues in mice, including the brain (Table 1) [39]. Their results revealed new possibilities for the direct delivery of macromolecules into patients.

3 Characteristics and Categories of PTD

Peptides capable of delivering macromolecules into living cells can be categorized as either "protein derived" or "designed" (Table 2) [40, 41]. Protein-derived peptides are short polypeptides encoded in natural proteins of various organisms and responsible for the penetration of proteins into cells. Tat-PTD derived from the HIV-1 Tat protein [23], penetratin from the homeodomain of *Drosophila* Antennapedia [28], pVEC from murine vascular endothelial cadherin [42], and signal sequence-based peptides from various cytokines [43] are categorized in this group. All the protein-derived PTDs share a "positive charge" caused by basic amino acids such as arginine and lysine residues. Such information has helped to the development of several potent synthetic peptides such as polyarginine and polylysine, which show potential for penetration [44]. Amphipathic polypeptides

Table 2 Peptide sequences of representative PTDs

Category	Peptide	Peptide sequence
Protein-derived peptides	HIV-1 Tat peptide	YGRKKRRQRRR
	Penetratin (Ant)	RQIKIWFQNRRMKWKK
	pVEC	LLIILRRRIRKQAHAHSK
Designed peptides	R8 (Octaarginine)	RRRRRRRR
	K8 (Octalysine)	KKKKKKKK
	Transportan	GWTLNSAGYLLGKINLKALAALAKKIL
	MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV

are composed of hydrophobic and hydrophilic domains from different sources, such as transportan, comprising galanin fused to mastoparan [45], and MPG, comprising HIV-1 gp41 protein fused to a peptide from the nuclear localization signal of SV40 large T-antigen [46].

4 Mechanism of PTD-Mediated Protein Transduction into Living Cells

More than 100 reports concerning PTDs have appeared this decade, and significant progress has been achieved especially with regard to the molecular mechanisms underlying the internalization of both PTD peptides and PTD-conjugated macromolecules into living cells. It is widely accepted that electrostatic interaction of positively charged PTD with negatively charged cellular membrane is followed by three energy- and temperature-dependent processes, clathrin-mediated endocytosis, lipid raft-mediated caveolae endocytosis, or macropinocytosis (Fig. 1).

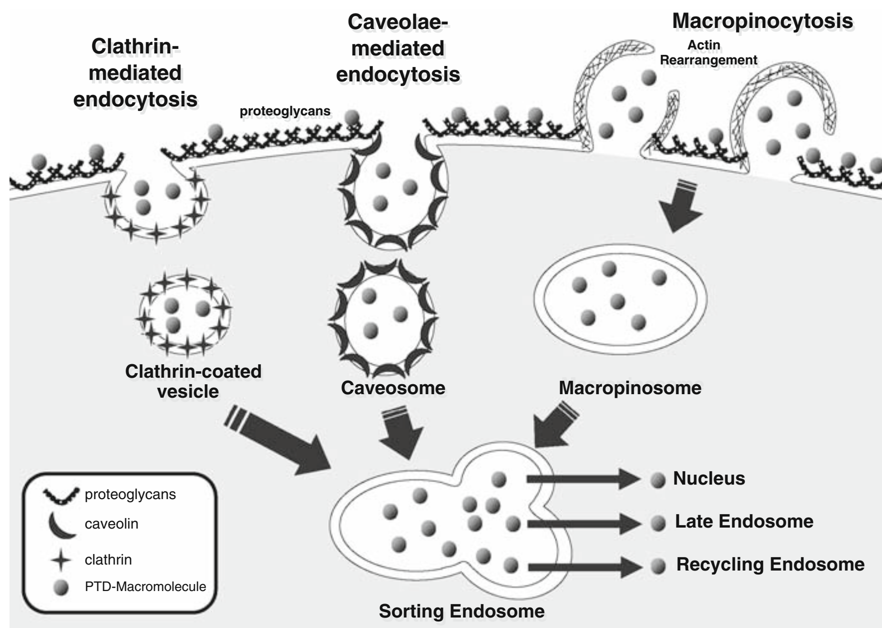

Fig. 1 Proposed model for the internalization of PTD-conjugated macromolecules into cells. Interaction of positively charged PTDs with negatively charged proteoglycans and glycosaminoglycans plays an important role in the internalization. The electrostatic interaction is followed by energy- and temperature-dependent endocytotic pathways. This involves phagocytotic and pinocytotic pathways: clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis

Early on, it was reported that the internalization of Tat protein occurred even at 4°C [23], and similar observations were reported for the basic amino acid-rich peptide derived from the antennapedia homeodomain [47]. Therefore, it had been widely assumed that the PTD-mediated internalization occurs in an energy- and receptor-independent manner and is alternatively based on direct transport through the lipid bilayer. However, it has been reported that the energy independence and receptor independence resulted from experimental artifacts in the process of cell fixation prior to microscopic observation and also were due to the inadequate removal of PTD conjugates bound to the cell surface [48, 49]. Moreover, it has been reported that the internalization is almost completely suppressed at 4°C in unfixed conditions [49, 50]. All of these recent results, together with the observation that heparan sulfate and an inhibitor of low-density lipoprotein receptor-related protein precluded the cellular uptake of PTD peptides and PTD-fused macromolecules [50, 51], suggest that the interaction of positively charged PTDs with negatively charged cell surface constituents, such as proteoglycans and glycosaminoglycans (heparan sulfate, heparin), plays an important role in the internalization (Fig. 1) [51–54]. Also, the electrostatic interaction is followed by the three energy- and temperature-dependent processes [48].

A characteristic of clathrin-mediated endocytosis is the formation of clathrin-coated membrane pits that pinch off the cellular membrane to generate vesicles [49]. The involvement of clathrin-mediated endocytosis in the internalization of PTDs was suggested by the finding that an FITC-labeled avidin–Tat-PTD complex colocalized with transferrin, a classical endocytic marker [55]. The importance of the classical endocytotic pathway and the temperature sensitivity of PTD internalization was confirmed further [48].

A typical feature of caveola-mediated endocytosis is the formation of non-coated invaginations composed of detergent-resistant membrane components rich in cholesterol and sphingolipids, known as lipid rafts [56]. The importance of the caveola-mediated mechanism in the PTD-mediated internalization was confirmed in an experiment where the cellular uptake of Tat peptide was affected by drugs that either disrupt lipid rafts or alter caveolar trafficking [57]. Moreover, Tat-PTD-fused protein showed colocalization with a marker of caveolar uptake, caveolin, further strengthening the importance of the mechanism in the PTD-mediated internalization [57].

The importance of macropinocytosis in the PTD-mediated internalization of macromolecules was reported recently [58, 59]. Macropinocytosis defines a series of sequential events: (1) a dramatic reorganization and ruffling of the plasma membrane by actin filaments, (2) the formation of an external macropinocytic structure, and (3) the inclusion and internalization of large vesicles, known as macropinosomes, into the cytoplasm [60]. Using a permeable Tat-Cre recombinase reporter and living cells, Wadia et al. demonstrated that Tat-fusion proteins are rapidly internalized by the macropinocytic pathway after the initial ionic cell–surface interaction, confirming the importance of macropinocytosis for the internalization of macromolecules [58].

It is generally accepted that the mechanism responsible for the internalization depends on the size of the PTD conjugate. Namely, the macropinocytic pathway seems to be responsible for the internalization of macromolecules. In addition, caveolae-mediated pathway has also been reported to play an important role in it [57]. On the other hand, the caveolae-mediated or clathrin-mediated endocytic pathways seem to function in the internalization of PTD itself or PTD-conjugated small molecules.

5 Kinetics and Tissue Distribution of PTD-Fused Protein in Living Animals

Kinetics and tissue distribution of PTD-fused protein drugs in vivo are important information for the development of novel PTD-mediated anticancer therapeutics and for the optimization of therapeutic regimens. Schwarze et al. demonstrated that biologically active proteins could be delivered to many tissues including the liver, kidney, heart, muscle, lung, spleen, and brain of mice after intraperitoneal injection of 200–500 μg of Tat–β-Gal fusion protein [39]. Polyakov et al. reported a preliminary but important information that intravenous injection of Tat-PTD labeled with Technetium-99m (^{99m}Tc) showed a rapid distribution to whole body [61]. The level of Tat-^{99m}Tc in organs reached peak within 5 min after injection and showed modestly rapid blood clearance. The Tat-^{99m}Tc was rapidly cleared by both renal and hepatobiliary excretion over the subsequent 2 h with activity appearing in the urinary and bladder and bowel. In addition, Cai et al. analyzed in detail when Tat–β-Gal fusion protein is distributed in different organs, such as liver, kidney, spleen, lung, bowel, and brain, through four different routes, such as portal vein, intravenous, intraperitoneal, and oral administration [62]. Tissues were harvested 15 min, 1 h, 6 h, 10 h, and 24 h after the administration and subjected to enzymatic activity assay. β-Gal activity peaked at 15 min in most tissues after portal vein, intravenous, and intraperitoneal administration and at 1 h after oral dosing in all tissues (Fig. 2). β-Gal activity in the liver at 15 min after portal vein injection was higher than after intravenous, intraperitoneal, and oral dosing. The median initial half-life for activity was 2.2 h, ranging from 1.2 h to 3.4 h (Table 3). All of these pharmacokinetic data allow rational optimization of delivery route and schedules for therapeutic PTD-fused proteins.

6 Development of PTD-Mediated Anticancer Protein Drugs

Research on protein transduction has dramatically expanded from in vitro to in vivo in the last decade. The advantage of this application is that one can accomplish the rapid and equal distribution of PTD-conjugated macromolecules to all tissues and cells in vivo [39]. However, as medications, PTD-fused

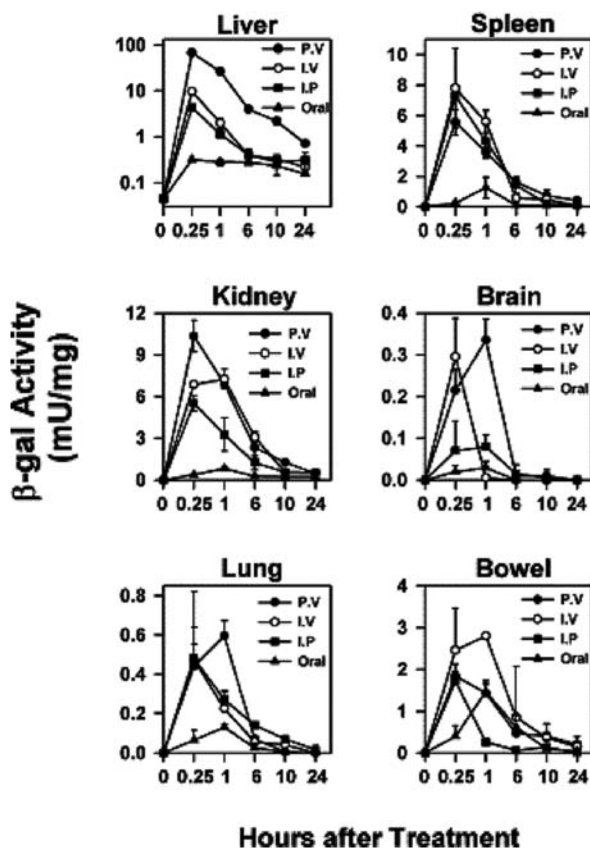

Fig. 2 Quantitative analysis of Tat–β-Gal activity in organs. Tat–β-Gal fusion protein was injected through four routes (portal vein, intravenous, intraperitoneal, oral), and β-Gal activity of homogenates was normalized to the protein concentration in the sample. This figure was derived from Ref. [62]; therefore, see Ref. [62] for details

Table 3 Half-life of β-galactosidase activity in the liver, kidney, and spleen of a mouse after administration

	Half-life of β-Gal in each tissue after administration (500 μg/mouse)		
Route of administration	Liver (h)	Kidney (h)	Spleen (h)
Portal vein	1.4	2.9	2.9
Intravenous	1.2	3.4	1.9
Intraperitoneal	1.7	2.9	2.3

The table was derived from Ref. [62]; therefore, see Ref. [62] for details.

anticancer macromolecules should have target specificity and act locally; otherwise damage to normal tissues and side effects may occur. In the following sections, we focus on the use of PTDs to develop anticancer macromolecules and introduce several representative strategies to distinguish between tumors and normal tissues to increase specificity but decrease side effects (Table 4).

Table 4 PTD-mediated anticancer strategies

Cargo	Effect	PTD	References
ODD-procaspase3	Tumor growth delay, radiosensitization	Tat	4–6, 78
p53 C-terminal region (p53C)	p53-dependent apoptosis	pAntp	7
p53C′ (modified p53C)	Tumor growth delay, 6 times extension in lifespan	Tat	8
p53 N-terminal region (p53N)	Activation of apoptotic genes, cytotoxicity for cancer cells, regression of human retinoblastoma cells in a rabbit eye	Tat	9
SmacN7	Enhancement of TRAIL in a tumor xenograft	Tat	10
SmacN7	Reversion of apoptosis resistance, synergistic effect with a chemotherapy	R8	11
Surv–T34A (Survivin T34A)	DNA fragmentation, aberrant nuclei formation, tumor cell apoptosis, tumor growth delay, 40–50% reduction in tumor mass	Tat	12
BH3 domain of Bak	Apoptosis of cancer cells	pAntp	13
BH3 domain of Bim	Apoptosis of cancer cells, tumor growth delay	Tat	14
$VHL_{104\text{-}123}$	Inhibition of thymidine incorporation into RCC cells, inhibition of MAP kinase pathway, inhibition of RCC proliferation in vitro, growth delay of RCC xenografts, regression of tumor volume, inhibition of invasion of RCC tumor	Tat	15
ESX	Reduction in ErbB2 protein level, induction of apoptosis retardation of growth of ErbB2-overexpressing breast cancer cells,	Tat	16
Tumor-associated antigen: OVA	Antigen presentation to CD4 T-cells, tumor regression in mice	Tat	17

6.1 *Application of a HIF-1α ODD Domain; Development of Hypoxia-Targeting Protein Drugs*

Genetic alterations directly cause the deregulated proliferation and high metabolic demands of tumor cells, which in turn lead to the imbalance of tumor growth and the development of a tumor vasculature. These phenomena consequently cause hypoxic areas in solid tumors, to which the supply of oxygen from tumor capillaries is inadequate [63, 64]. Hypoxia has been recognized as a tumor-specific microenvironment; in other words, healthy adults probably have few hypoxic tissues. Under hypoxic conditions, a transcription factor, hypoxia-inducible factor-1 (HIF-1), induces the expression of various genes

related to angiogenesis [65] and glycolysis [66] and leads to invasive and metastatic properties of tumor cells [67]. Moreover, HIF-1 activity is associated with the resistance of tumor cells to conventional radiotherapy and chemotherapy [68, 69] and with patient mortality in clinical studies [70–72]. Therefore, extensive efforts have been devoted to the development of novel therapies, which specifically damage hypoxic/HIF-1-activating tumor cells [69, 73].

HIF-1 is a heterodimeric transcription factor composed of an alpha subunit (HIF-1α) and a constitutively expressed beta subunit (HIF-1β) [74]. HIF-1 activity is mainly dependent on the level of HIF-1α protein [74, 75]. Under hypoxic conditions, HIF-1α interacts with HIF-1β and functions as a transcription factor. Under normoxic conditions, the oxygen-dependent degradation (ODD) domain of HIF-1α is hydroxylated by prolyl hydroxylases (PHDs) and ubiquitinated by the von Hippel–Lindau (VHL) tumor suppressor protein-containing E3 ubiquitin ligase, resulting in rapid degradation of the HIF-1α protein [76, 77].

We took advantage of this unique property of the ODD domain to develop a novel hypoxia-targeting protein drug [4–6, 78]. First of all, we identified the minimum region of the ODD domain responsible for the oxygen-dependent degradation of arbitrary proteins fused to it [4]. We confirmed the hypoxia-dependent β-Gal and luciferase activity of ODD–β-Gal fusion protein [4] and ODD–luciferase fusion protein [79], respectively, in cultured cells. To apply the ODD-fusion protein to an in vivo study, we fused Tat-PTD to the N-terminal of the ODD–β-Gal protein and created a Tat–ODD–β-Gal triple-fusion protein [4]. After i.p. injection with the Tat–ODD–β-Gal fusion protein into subcutaneous tumor-bearing mice, β-Gal activity was detected only in the hypoxic regions of the solid tumor, not in normal tissue [4]. These results demonstrate that biologically active proteins can be exogenously delivered to hypoxic tumor cells by the Tat–ODD peptide in vivo. This was the first example of the target specificity of Tat-mediated protein delivery. To examine whether the Tat–ODD fusion protein with cytotoxicity shows a hypoxia-targeting effect, the Tat–ODD peptide was fused to a proapoptotic protein [4–6, 78]. We intentionally chose a precursor of caspase-3, procaspase-3, because it is activated in response to hypoxic stress, which was thought to reduce the possibility of side effects in the well-oxygenated normal tissues (Fig. 3A) [4, 78]. Systemic administration with the resultant fusion protein, Tat–ODD–procaspase-3 (TOP3), dramatically induced apoptosis of tumor cells in the border area between well-oxygenated viable cells and necrotic cells (Fig. 3B) [5]. TOP3 reduced volume of hypoxic tumor cells and entire tumor itself, as well as suppressed tumor growth without any obvious side effects (Fig. 3C) [4–6]. The hypoxia-targeting effect of TOP3 was proven using a rat ascites model, in which the intraperitoneal injection of MM1 cells results in highly hypoxic ascetic fluid [80]. Inoue et al. demonstrated that the intraperitoneal injection of TOP3 resulted in a significant increase in the lifespan of rats with malignant ascites, and furthermore, 60% of the treated animals were cured without the recurrence of ascites.

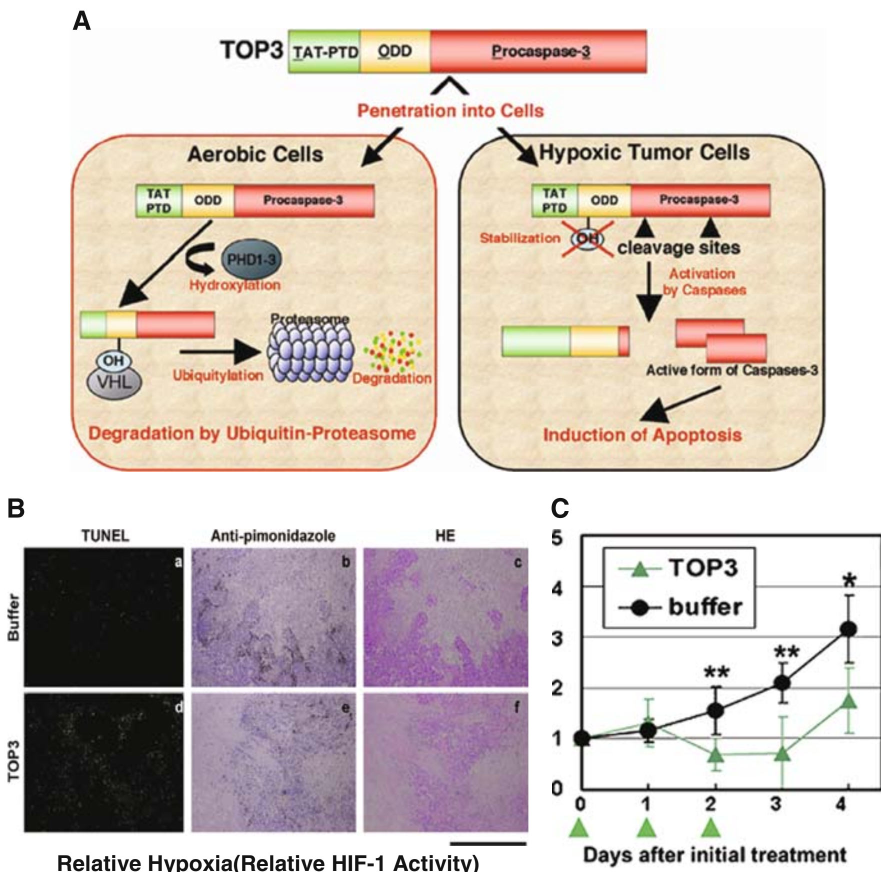

Fig. 3 TOP3 specifically targets HIF-1-active (hypoxic) tumor cells. (**A**) PTD facilitates internalization of TOP3 into tumor cells. In normoxic tumor cells, TOP3 is hydroxylated and ubiquitinated through the same regulation as HIF-1α protein, and resultantly degraded by 26S proteasome. Meanwhile, in hypoxic tumor cells, TOP3 is activated and induces apoptosis. See Ref. [78] for details. (**B**) TOP3 increased TUNNEL-positive apoptotic cells in border area between normoxic and necrotic areas. See Ref. [5] for details. (**C**) TOP3 significantly reduces HIF-1-positive (hypoxic) tumor cells. See Ref. [6] for details. This figure was modified from Ref. [5, 6, 78]

6.2 Restoration and Activation of p53 Function

Genetic alterations of oncogenes and/or tumor suppressor genes cause deregulated proliferation and the evasion of apoptosis and consequently make cells more malignant [1]. In the development of novel cancer therapy strategies, extensive efforts have been devoted to restoring the lesions preventing the implementation of the apoptotic response and leading to the death of malignant

cells. Such treatments are expected to be much less toxic to normal tissue than the conventional genotoxic agents currently in clinical use.

The gene encoding the tumor suppressor p53 is the most common antiapoptotic lesion in cancer cells [81], and more than 50% of human cancers have been reported to harbor *p53* gene mutations. In most remaining cases, p53 activity is impaired by alternative molecular mechanisms, such as an elevated level of a p53 inhibitor, Mdm2 [82], and the E6 protein of HPV [83], or silencing of a p53 coactivator, ARF [84, 85]. One of the most important functions of p53 is "cell cycle arrest," in which p53 disturbs the replication of damaged genomic DNA and the fixation of mutations, allowing for DNA repair. Another important function is the "induction of apoptosis," which occurs when the damage to the genomic DNA is too severe to be repaired. Both of these functions are essential for the regulation of cell proliferation in multicellular organisms [85], and their loss frequently leads to cellular neoplastic transformation and increases the resistance of cancer cells to anticancer therapies [86]. Therefore, restoring p53 function in tumor cells has been recognized as an effective way to induce cancer cell death in a large population of cancer patients. Gene therapy strategies with viral or nonviral vectors have been used to introduce a functional p53 gene into experimental tumor xenografts; however, the transfer efficacy did not meet our demands [87]. Additionally, a problem associated with immunogenicity was inevitable with this drug delivery system [88]. To overcome these difficulties, Tat-mediated approaches were carried out as follows.

Selivanova et al. fused the Antennapedia transduction domain to a p53 C-terminal peptide (p53C), which was previously reported to activate the function of both wt p53 and several mutant forms of p53, and it confirmed that the fusion protein induced p53-dependent apoptosis in several tumor cell lines [7]. On the other hand, the fusion protein had no apparent apoptosis-inducing effect on normal cells with a functional *p53* gene. Snyder et al. then introduced a structural modification into the C-terminal to increase the stability of the protein, fused the resultant p53C' to Tat-PTD, and introduced the fusion protein (Tat-p53C') into tumor-bearing mice [8]. After an efficient delivery of Tat-p53C' to all the cells in mice, the protein was activated in the tumor xenograft but not in normal tissue. Moreover, intraperitoneal injection of the protein for 12 consecutive days caused a significant reduction in tumor growth and a 6-fold extension of lifespan.

Harbour et al. reported another strategy targeting *p53* lesion. They also aimed to restore endogenous p53 activity by using a permeable peptide [9]. HDM2 is known to decrease the ability of p53 to function as a positive transcription factor and facilitate proteolytic degradation of the protein through direct interaction with its N-terminal region [89]. Indeed, the overexpression of HDM2 has been reported in many clinically recognized tumors, which contain the wild-type *p53* gene and is associated with the functional inactivation of the p53 protein [90–92]. Therefore, the disruption of the inhibitory effect of HDM2 on p53 activity would be expected to yield therapeutic benefits in tumor cells that overexpress the HDM2 protein. Consequently, the N-terminal region of the p53 protein was fused to

Tat-PTD. The resultant Tat-p53N peptide induced the rapid accumulation of p53 and the activation of apoptotic genes, and it resulted in the preferential killing of tumor cells and the regression of human retinoblastoma cells in rabbit eyes [9]. Minimal retinal damage was observed after intravitreal injection [9].

6.3 Modification of Apoptotic Pathway

A major obstacle in cancer therapy is the resistance of cancer cells to current anticancer treatments: chemotherapy and radiation therapy [93]. Defects in apoptotic programs, which are caused by deregulated expression and function of the components of the apoptotic pathway, contribute to such resistance [94, 95].

Inhibitors of apoptosis proteins (IAPs), which inhibit caspase activity by directly binding to activated caspase-3 and -7, are frequently overexpresssed in malignant tumors [96]. The second mitochondria-derived activator of caspases (Smac) is an important factor that is released from the mitochondria to the cytosol, antagonizes IAPs, and release caspases to promote apoptosis [97, 98]. Based on these mechanisms, one can expect the upregulation of Smac activity in tumor cells to improve resistance to anticancer therapies. Fulda et al. examined this hypothesis using a cell-permeable synthetic peptide composed of Tat-PTD and seven N-terminal amino acids of the Smac protein (AVPIAQK) [10]. The cell-permeable Smac polypeptide was expected to inactivate X-linked IAP (XIAP), disrupt the interaction of XIAP and caspase-9, and consequently induce apoptosis. The peptide enhanced the therapeutic effect of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in an intracranial malignant glioma xenograft model [10]. Moreover, the complete eradication of established tumors was achieved only upon combined treatment with the Smac peptide and Apo2L/TRAIL. In these experiments, no detectable toxicity to normal brain tissue was observed.

Yang et al. examined whether the inhibition of IAPs combined with chemotherapy produced synergistic effects [11]. First of all, they confirmed that the defect in apoptosome activity was dramatically restored by the same seven amino residues of Smac protein through the disruption of XIAP–caspase-9 interaction. On the other hand, SmacN7 peptide did not have any striking effect on the apoptosome activity of normal lung fibroblast cells. They further demonstrated that newly synthesized SmacN7 peptide fused to the cell membrane-permeable polyarginine (SmacN7R8) strongly reversed the resistance to apoptosis, and it displayed a synergistic effect with chemotherapy in vivo (Fig. 4).

Because Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is highly expressed in most tumors, it has been recognized as a promising therapeutic target in cancer [99]. A variety of Survivin antagonists have been reported to rescue the apoptotic pathway and induce apoptosis in

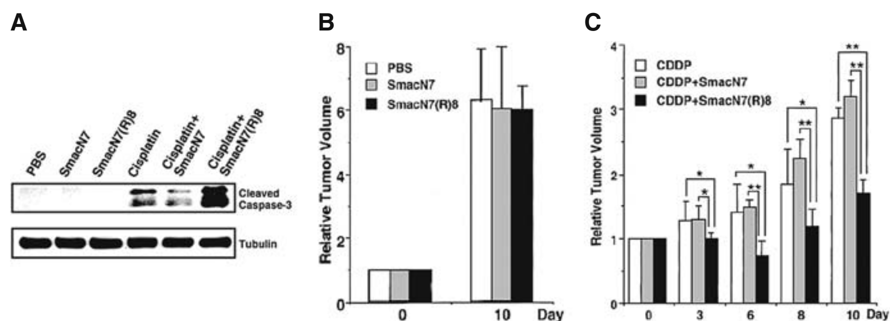

Fig. 4 Effect of SmacN7R8 in combination with chemotherapeutic agent on tumor growth in tumor-bearing mice. Human nonsmall-cell lung carcinoma, NCH-H460, tumor-bearing mice were treated with PBS, SmacN7 (without cell permeability), SmacN7R8 (with cell permeability), Cisplatin, Cisplatin + SmacN7, or Cisplatin + SmacN7R8. (**A**) Western blotting for cleaved caspase-3 in the tumor tissues at 48 h after the treatment. (**B and C**) Tumor volume at indicated days after the treatment. Note: Cell permeable SmacN7R8 sensitized the apoptosis-inducing activity of Cisplatin and potentiated the antitumor activity of Cisplatin. This figure was modified from Ref. [11]; therefore, see Ref. [11] for details

malignant cells; however, the utility of these agents has been limited by the inadequate delivery and permeability into tumor cells. To overcome these problems, Yan et al. generated a recombinant fusion protein composed of Tat-PTD and a dominant-negative mutant of Survivin, T34A mutant [12]. Tat–Surv–T34A induced cell detachment, DNA fragmentation, caspase-3 activation, and mitochondrial release of apoptosis-inducing factor in vitro. Moreover, intraperitoneal injection of the fusion protein into subcutaneous tumor-bearing mice increased aberrant nuclei formation and tumor cell apoptosis, resulting in a 40–50% reduction in growth and mass of the tumor xenografts.

A characteristic of the *Bcl-2* family is the presence of one or more conserved Bcl-2 homology domains (BH1-4) corresponding to four α-helical segments of Bcl-2 [100]. The *Bcl-2* family is categorized into three groups: antiapoptotic proteins (Bcl-2, Bcl-xL, Mcl-1), BH3-only proteins (Bim, Bid, Bad, PUMA), and BH1-3 multidomain proteins (Bax, Bak, Bok). The BH3-only proteins bind to the BH-4 domain of the antiapoptotic proteins, antagonize the function of these proteins, and consequently induce apoptosis of cancer cells. Applying this molecular mechanism, Holinger et al. fused the antennapedia PTD to the BH3 domain and successfully induced the apoptosis of HeLa cells [13]. Similarly, Kashiwagi et al. synthesized a polypeptide composed of Tat-PTD and the BH3 domain of Bim and confirmed that Tat–Bim induced apoptosis of T cell lymphoma (EL4), pancreatic cancer (Panc-2), and melanoma (B16) cells in a dose-dependent fashion [14]. Moreover, local injections of Tat–Bim twice a day for 7 days significantly delayed growth in murine models of pancreatic cancer and melanoma.

6.4 Modification of IGF-I Signaling Pathway

The growth of various kinds of cancer cells depends on insulin-like growth factor-I (IGF-I)-mediated signaling; therefore, inhibiting this pathway has been recognized as a promising strategy. Indeed, direct inhibition of this pathway using a truncated form (dominant-negative form) of the IGF-I receptor (IGF-IR) [101], a specific IGF-IR antibody [102], or a specific IGF-IR antisense oligonucleotide [103] showed a significant therapeutic effect on various experimental tumor xenografts. In addition to such direct inhibition, an indirect method by transducing the functional VHL protein into cancer cells seems to be effective for renal cell carcinoma (RCC). This idea is attributed to the fact that VHL is functionally inactivated in many RCCs [104], and the dysfunction accelerates the growth of RCC cells through the activation of the IGF-I-mediated signaling pathway [15, 105]. Datta et al. fused Tat-PTD to a specific amino acid sequence of the VHL β domain ($VHL_{104\text{-}123}$), which binds to the cytoplasmic region of IGF-IR and inhibits IGF-I signaling, and examined the therapeutic effects on RCC [15]. The fusion protein, Tat–FLAG–VHL peptide, inhibited thymidine incorporation into RCC cells by nearly 80% compared with a counterpart protein (Tat–FLAG). Furthermore, the Tat–FLAG–VHL peptide inhibited the tyrosine phosphorylation of MAP kinase, an essential downstream molecule that leads to cell proliferation. Thus, these results suggest that Tat– FLAG–VHL peptide blocks IGF-I-induced proliferation of RCC cells in vitro. Furthermore, i.p. injections of Tat–FLAG–VHL peptide retarded the growth of subcutaneous RCC tumors, and in some cases, reduced the tumor volume, and dramatically inhibited invasiveness into the muscle layer.

6.5 Modification of ErbB2 (HER-2/neu) Expression

The *ErbB2 (HER-2/neu)* gene is a member of the epidermal growth factor receptor family and is overexpressed in ~30% of breast cancers [106, 107]. Deregulated expression of this gene is associated with lymph node metastasis and a poor prognosis [108, 109]. Therefore, the ErbB2 would serve as an excellent target for the development of novel cancer treatments. One of the critical transcription factors that activate the ErbB2 expression in breast cancer is ESX (ESE-1//ELF3/ERT/Jen) [110]. ESX interacts with DRIP130/CRSP130/Sur-2, a Ras-linked metazoan-specific subunit of human mediator complexes, binds to the ESX-binding site in the *ErbB2* promoter, and activates the transcription of the *ErbB2* gene [16]. Disruption of ESX–DRIP130 interaction is reported to impair *ErbB2* gene expression and reduce the proliferation and viability of *ErbB2*-expressing breast cancer cells [16]. Asada et al. identified the region essential for ESX–DRIP130 interaction and designed a cell-permeable form of ESX peptide using Tat-PTD [16]. The Tat–ESX peptide reduced the ErbB2 protein level, retarded cell growth, and

induced apoptosis in ErbB2-overexpressing breast cancer cells. The important point of their report is that cells with low ErbB2 protein levels were insensitive to the Tat–ESX peptide.

6.6 Application to Dendritic Cell (DC) Vaccines

Dendritic cell (DC)-based vaccines are being developed to treat cancer, and clinical trials are ongoing [111, 112]. A primary goal of the strategy is to elicit responses from cytotoxic lymphocytes (CTL) that can kill tumor cells. In most cases, tumor antigen-derived peptides are loaded into DCs in vitro, and the cells are administered into patients. Expression of the defined tumor antigen in DCs can be achieved by transfecting the cells with cDNA encoding the tumor antigen or by infecting the cells with virus expressing the antigen; however, there are two problems with these methods. First, the transfection of DCs is inefficient, and second, there are practical and theoretical concerns that relate to the use of viral vectors in patients. As an alternative to these genetic modifications, Shibagaki et al. explored PTD-mediated antigen transduction into DCs to elicit CTLs that can lead to tumor rejection in vivo [17]. They demonstrated that a bacterial recombinant model tumor-associated antigen that was fused to Tat-PTD was efficiently transduced into murine lymphocytes and DCs and was processed by proteasomes. The resultant peptides were displayed on the cell surface bound to MHC class I. The transduced DCs were able to elicit CTLs in vivo, and the CTL activity was sufficient to both prevent engraftment with antigen-expressing tumors into mice and lead to a partial regression of the established tumor mass.

6.7 PTD-Mediated Modulation of Deregulated Cell Cycle of Cancer Cells

In nonmalignant cells, the product of the *Rb* gene (pRb) binds to transcription factors belonging to the E2F family and represses their function as transcriptional activators [113]. Cyclin-dependent kinases (Cdks) inactivate pRb, release E2F from the pRb–E2F complex, and consequently upregulate the transcription of late G1 phase-specific genes responsible for S-phase entry. The Cdks are known to be activated through the formation of a complex with cyclins (A–D) and inactivated by p16INK4a, p21, and p27 [114, 115]. This well-organized regulation of the cell cycle is disrupted in malignant cells through genetic alterations, such as inactivation of p16INK4a, amplification of cyclin D1 or Cdk4, or loss or mutation of *Rb gene* [116, 117]. Therefore, reconstitution of the tumor suppressor function of p16INK4, p21, and p27 has been an aim for cancer therapy. As for p16INK4, it has been reported that a 20-amino acid poly peptide in the third ankyrin-like repeat of p16 is sufficient to inhibit cyclin D-Cdk4/6-dependent inactivation of pRb [118]. Based on this knowledge, Guis et al. demonstrated that this p16 polypeptide fused to Tat-PTD (Tat-p16

peptide) was internalized into cultured cells and subsequently induced G1 arrest [119]. As for p21, Ball et al. demonstrated that a 20-amino acid peptide of the C-terminal Cdk-binding domain of p21 conjugated with antennapedia PTD suppressed pRb phosphorylation (inactivation) and consequently induced G_1/S cell cycle arrest (Fig. 5) [120]. As for p27, in order to reconstitute its tumor suppressor function, it was fused to Tat-PTD and applied to human Jurkat

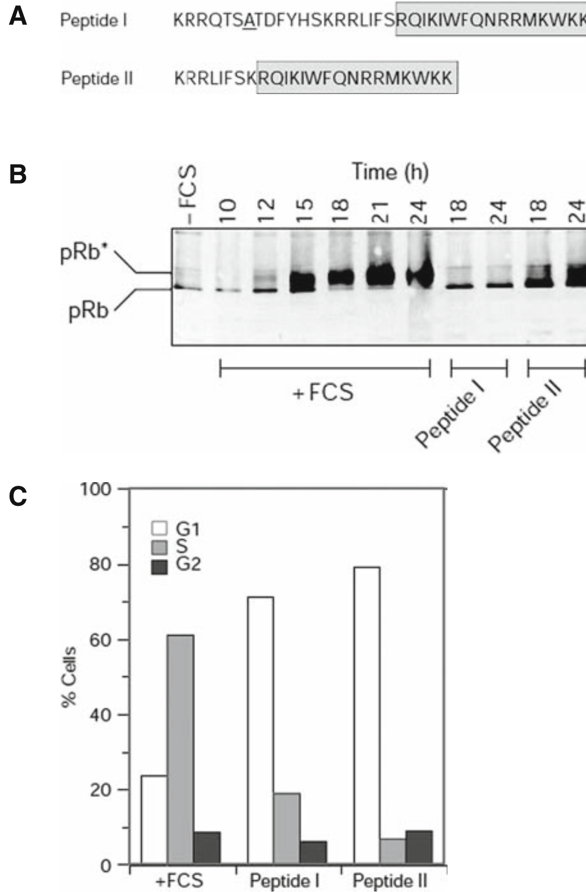

Fig. 5 C-terminal Cdk-binding domain of p21 conjugated with antennapedia PTD suppresses pRb phosphorylation and induces G_1/S cell cycle arrest. (**A**) Peptide sequences of fusion polypeptides composed of C-terminal Cdk-binding domain of p21 ($p21_{141\text{-}160}$ [peptide I]; or $p21_{154\text{-}160}$ [peptide II]) and antennapedia PTD (*boxed*). (**B**) pRb became hyperphosphorylated between 12 and 15 h after serum was added to starved HaCat cells, but in the presence of peptide I or II remained hypophosphorylated. pRb = hypophosphorylated, pRb* = hyperphosphorylated. (**C**) Cell cycle distribution of HaCat cells after culture in D-MEM medium containing 10% FCS alone or 10% FCS with peptide I or II. Note: Peptide I and II inhibited the phosphorylation of pRb and induced G_1/S cell cycle arrest. This figure was modified from Ref. [120]; therefore, see Ref. [120] for details

T cells in vitro [121]. The Tat-p27 protein dose-dependently induced cell cycle arrest at G1. Snyder et al. demonstrated that the Tat-p27 tumor suppressor protein actually inhibited tumor growth in two mouse models, such as a H1299 subcutaneous solid tumor xenograft model and a more clinical-relevant peritoneal tumor model [122].

6.8 Specific Delivery of PTD-Conjugated Macromolecules to Cancer Cells

Several in vitro studies have reported other possibilities which may enable the delivery of the PTD-conjugated macromolecule specifically to the desired tumor in vivo. First, because tumor cells are reported to have unique glycosaminoglycan on their surface, PTDs, which selectively interact with it, may enables us to selectively target tumor cells [55]. Second, by inserting a tissue- and organelle-specific cleavage recognition site between it and the macromolecule, PTD may be cleaved off, resulting in the accumulation of the PTD-free macromolecules in the desired tissue and organelle [123]. Third, it is also possible to generate a PTD-linked protein drug that specifically acts on tumor cells without affecting normal cells, by applying transformed cell-specific protein activity. Finally, by using a peptide that can be recognized by the tumor-specific membrane proteins, it may be possible to design a variety of proteins that are specifically internalized into desired tissues.

7 Conclusions and Perspectives

Recent advances in molecular and cellular biological techniques have helped to reveal the mechanisms underlying carcinogenesis and tumorigenesis. Based on this knowledge, several ways of discriminating nonmalignant and malignant cells have been proposed, in order to develop PTD-mediated anticancer strategies. Good examples are the exploitation of tumor-specific phenomena, such as dysfunction of p53, suppression of the apoptotic pathway, accelerated IGF-I or ErbB2 (HER-2/neu) signaling pathway, or tumor hypoxia. Each strategy actually showed expected anticancer effects; however, all of them have been achieved only in animal experiments. We are now confronted with the task of applying these strategies to the clinical setting. For that purpose, we cannot ignore the following problems: how to decrease the immunogenicity of PTD-conjugated macromolecules, and how to prepare enough high-quality PTD-conjugated macromolecule. Overcoming these problems should lead to the development of a new generation of anticancer strategies. In addition, the continuation of efforts to explore novel molecular mechanisms responsible for cancer-specific phenomena will pave the way to the development of an innovative PTD-mediated cancer therapy.

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