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Review

Antiapoptotic Fusion Protein Delivery Systems

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Abstract: Apoptosis is a natural cell suicide mechanism to maintain homeostasis. However, many of the diseases encountered today are caused by aberrant apoptosis where excessive apoptosis leads to neurodegenerative disorders, ischemic heart disease, autoimmune disorders, infectious diseases, etc. A variety of antiapoptotic agents have been reported to interfere with the apoptosis pathway. These agents can be potential drug candidates for the treatment or prevention of diseases caused by dysregulated apoptosis. Obviously, world-wide pharmaceutical and biotechnology companies are gearing up to develop antiapoptotic drugs with some products being commercially available. Polymeric drug delivery systems are essential to their success. Recent R&D efforts have focused on the chemical or bioconjugation of antiapoptotic proteins with the protein transduction domain (PTD) for higher cellular uptake with antibodies for specific targeting as well as with polymers to enhance the protein stability and prolonged effect with success observed both *in vivo* and *in vitro*. All these different fusion antiapoptotic proteins provide promising results for the treatment of dysregulated apoptosis diseases.

Keywords: apoptosis, antiapoptotic fusion protein, drug delivery system.

Introduction

Apoptosis is a genetically regulated programmed cell death which exists in all multicellular organisms. This process can be triggered by external factors such as exogenous oxidative stress (ischemia),[1] nitric oxide,[2] serum or glucose deprivation,[3] as well as internal factors which include toxic substances (mutated SOD1) and byproducts of cell metabolism. These factors will cause cells to undergo a variety of morphological changes, including blebbing, changes in the mitochondrial membrane potential, PS translocation, cell shrinkage, nuclear fragmentation, chromatin condensation, dilated endoplasmic reticulum and chromosomal DNA fragmentation.[4-6]

Generally, the apoptosis involves the activation of caspases which belong to the class of cysteine proteases. Once the caspase was activated, it will initiate caspase cascades and finally resulting in cellular apoptosis.[7] The regulation of apoptosis follows two major pathways; the mitochondria regulated intrinsic pathway and the extrinsic pathway (Figure 1).

Extrinsic Apoptosis Pathways (Type I and Type II). A cell surface receptor named "death receptor" belongs to a part of the tumor necrosis factor (TNF) superfamily which functions in transmitting apoptotic signals after ligation with specific ligands. Up to date, the known death receptors

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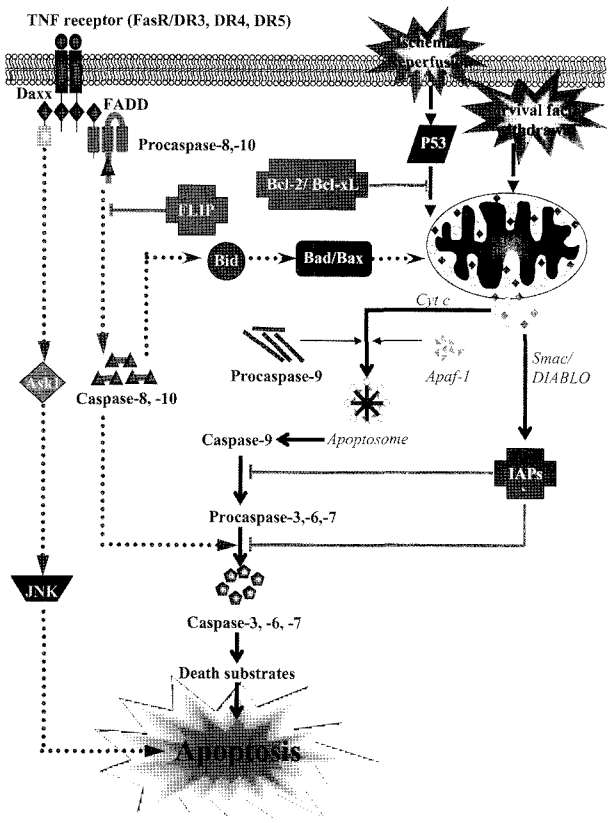

Figure 1. Apoptosis pathway. Dotted line indicates Extrinsic apoptosis pathways (Type I and Type II) while solid line indicates Intrinsic apoptosis pathway.

are Fas (CD95 or Apo1), TNF-R1 (p55 or CD120a),[8-10] DR3 (Apo3, WSL-1, TRAMP, or LARD), TRAIL-R1 (APO-2 or DR4), TRAIL-R2 (DR5, TRAIL-R2, TRICK2, or KILLER), and DR6.[11-20]

In type I cell, upon the ligand-induced activation of death receptors, the death inducing signaling complex (DISC) is assembled and consequently activate a large amount of apoptosis-initiating proteases caspase-8 and caspase-10.[21] This in turn activates the cleavage of caspase-3 from procaspase-3 which leads to a caspase cascade independent of mitochondria apoptosis pathway.[22]

Type II cells, due to the weak signal from the activated receptor, can not generate a caspase signaling cascade on its own. The activation of mitochondrial pathway is initiated when the Bid, a Bcl-2 family member, is cleaved by caspase-8. The truncated Bid translocates to the mitochondria and causes loss of mitochondrial transmembrane potential and subsequently releases cytochrome *c* and other apoptogenic factors into the cytoplasm of the cell.[23] Cytochrome *c* binds to Apaf1, recruits dATP to form a correct apoptosome complex and oligomerizes pro-caspase-9.[24] Activated caspase-9 initiates a caspase cascade downstream involving caspase-3, caspase-7 and caspase-6 resulting cell death.[25]

Intrinsic Apoptosis Pathway. Mitochondria plays a central role as a regulator in the cell intrinsic apoptosis pathway which can be triggered by DNA damage, defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of survival factors or other types of severe cell distress and chemotherapeutic drugs.[26] This will result in the disruption of the mitochondrial inner transmembrane potential which will cause the release of proapoptotic molecules such as AIF (apoptosis inducing factor) which include SMAC/DIABLO and cytochrome *c* into the cytoplasm. Indeed, the regulation of cytochrome *c* release is controlled by both the Bcl-2 family proteins which act as inhibitors (Bcl-2, Bcl-x) or promoters (Bax, Bak, Bik) of apoptosis.[27] Cytochrome *c* interacts with Apaf1, activates caspase-9 which in turn, activates caspase dependent pathway as mentioned above. SMAC/DIABLO promotes apoptosis by binding to inhibitor of apoptosis (AIP) proteins to inactivate these factors from interfering caspase activation.[28,29]

Diseases Caused by Dysregulated Apoptosis. Cell homeostatis in human body is maintained when billions of cells are produced by mitosis and the same amount of cells die by apoptosis everyday.[30] Half of the human diseases are closely related to aberrant apoptosis,[31] where excessive apoptosis leads to neurodegenerative disorders such as Alzheimer's disease, ischemic heart disease, autoimmune disorders, infectious diseases including AIDS whereas insufficient apoptosis causes cancer, atherosclerosis, viral infections and premalignant disease.[32-34] To our concern, we will focus on the diseases caused by excessive apoptosis (Table I).[30]

In autoimmune disease, organ specific diseases are mostly characterized by T lymphocyte-mediated attack on the β-cells of the islets of Langerhans in insulin-dependent diabetes mellitus (IDDM), oligodendrocytes in the brain in multi-

Table I. Diseases Caused by Excessive Apoptosis

Neurodegenerative disorders *Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, spinal muscular atrophy*
Hematological disorders *Aplastic anemia, Fanconi anemia, Hodgkin's disease*
Autoimmune disorders *Autoimmune lymphoproliferative syndrome, Fulminant hepatitis, graft-versus-host disease, Hashimoto's thyroiditis, insulin-dependent diabetes mellitus, multiple sclerosis, rheumathoid arthritis*
Ischemic injury *Ischemia and reperfusion, kidney infarction, myocardial infarction, stroke*
Toxin-induced disease *Alcohol-induced hepatitis, pulmonary fibrosis, sepsis*
Bacterial and viral infection *Acquired immune deficiency syndrome (AIDS), Ebola virus, Chlamydia trachomatis, Helicobacter pylori, Neisseria meningitidis*
Miscellaneous *Traumatic spinal cord injury, tumor counterattack (immune privilege)*

ple sclerosis (MS) and thyrocytes in Hashimoto's thyroiditis. The Fas/Fas ligand system plays a main role in the destructive phase of these autoimmune disorders.[35]

Neurodegenerative disorders are mainly caused by neuronal death which involves a series of caspase activation.[36] Alzheimer's disease (AD) is caused by intracellular neurofibrillary tangle formation, extracellular deposition of β-amyloid, loss of synapses and neurodegeneration where the apoptosis is initiated when the caspase was activated by the precursor β-amyloid protein.[37] Huntingtons disease (HD) results from the translation from an aberrant expansion of CAG repeats into a polyglutamine repeat in Huntingtin protein (Htt), where Sanchez shown that Htt may results in the recruitment and activation of caspase-8.[38]

The key pathologic feature of acute myocardial infarction and heart failure is the induction of tissue damage by coronary artery occlusion due to apoptosis process.[39] Ischemia/reperfusion involves both mitochondrial pathway and the death-receptor pathway. Ischemia causes mitochondria losing the membrane potential as a result, proapoptotic factors are released into the cytoplasm and activate the caspase-3.[40] The Fas/Fas ligand-dependent mechanisms are also activated by ischemia causing metabolic derangements and ultimately cell death.[39]

Protein Transduction Domain (PTD)/Cell Penetrating Peptides (CPP). Protein transduction domain (PTD), also known as cell penetrating peptide (CPP), is a useful tool to cargo various molecules not only peptides, proteins, nucleic acids, but also various molecules with wide ranges of molecular sizes and physicochemical properties, such as low molecular weight drugs, non-covalent supramolecular complexes, magnetic beads, and even liposomes into the cells.[41-43] Therefore, it has become a powerful and potential gadget delivering therapeutic proteins/molecules into cells without significant damage to the plasma membranes or high toxicity to the cells.[44]

Among the peptide vectors, most typical PTD is a short segment derived from HIV-1 Tat which consists of 11 amino-acid residues. This arginine rich peptide domain has a basic structure which is responsible for nuclear localization. Schwarze *et al.* showed that Tat-fusion protein can cross the blood-brain barrier (BBB) into the brain via intraperitoneal injection.[45] Vives *et al.* reported that the critical component for translocation in Tat protein is caused by the arginine-rich segment.[46] Thus, proteins that consist of oligoarginine segment in their sequence were able to enter cells.[44] Wender *et al.* showed that the internalization of the 9-mer of arginine (R9) was 20-fold more efficient than Tat (49-57), and the D-arginine oligomer (r9) showed greater uptake rate enhancement (>100-fold) than L-arginine.[47] Han *et al.* also suggested that more than six arginine residues were required for efficient transduction of fusion proteins into the cells. They also discovered that other than arginine oligopeptides, lysine oligopeptide is also an active PTD which can efficiently transduce fusion protein into mammalian cells.[48] Other than the mentioned PTDs, penetratin,[49] polylysine,[50,51] HSV VP22,[52-54] nuclear localization signal (NLS)[55] and pep-1[56] have been also used successfully in protein transductions. Transduction of PTD-protein was proved to be dose and time-dependent.[57] PTD fusion proteins were shown to enter successfully *in vitro*[48,56] as well as crossing BBB *in vivo* studies.[45,58]

PTD-Antiapoptotic Protein. *In vitro* studies with PTD fusion protein demonstrated efficient transduction into all cell types by direct addition to the cell culture medium.[59,60] In addition, *in vivo* studies showed that all cells within the body including those protected by blood-brain barrier were targeted.[45] PTD fusion protein has gained its popularity in recent years over the traditional gene therapy using viral carriers which cause serious toxicity and immunogenicity. The use of PTD for the introduction of therapeutic proteins, peptides, and siRNAs may be able to evade the drawbacks of gene therapy. In this review, our focus was on the therapeutic drugs for the treatment of dysregulated apoptosis diseases and below is a summarized table on the recent publications on PTD fused with antiapoptotic proteins (Table II).

Bcl-x_L is an antiapoptotic member which belongs to Bcl-2 pro-survival proteins possessing BH1, BH2, BH3 and BH4 domains.[72] It potently inhibits apoptosis in response to the release blockage of cytochrome *c* from mitochondria and inhibits the caspase independent apoptosis pathway.[73] Cao *et al.* studied Tat-HA-Bcl-x_L fusion protein by intraperitoneal injection into mice and the cerebral infarction was decreased up to 40% in a dose-dependent manner.[58] Ischemia-induced caspase-3 activation in ischemic neurons has been successfully attenuated by TAT-HA-Bcl-x_L. In cerebellar granule cells *in vitro* studies, Dietz *et al.* showed that Tat-Bcl-x_L completely blocked low-potassium-induced apoptosis while 24% of mouse retinal ganglion cells was prevented from optic nerve lesion caused retrograde neuronal apoptosis through intraocularly injection. 70 Tat-Bcl-x_L and Tat-BH4 are studied by Hotchkiss *et al.* in an *ex vivo E. coli*-induced human lymphocyte apoptosis model and *in vivo* mouse model of sepsis and the lymphocyte apoptosis of both model has been prevented and markedly decreased respectively.[67] PTD conjugated Bcl-x_L fusion protein may be a potential therapeutic drug for the treatment of neurological disorders, cerebral ischemia as well as sepsis.

Since a number of publications have demonstrated that the PTD-fusion proteins were capable of crossing the BBB, it provides a great chance for the delivery of therapeutic drugs to the brain.[45,63,69] Lately several trials have been proved to overcome brain diseases by conjugating PTD with neurotrophic factor either derived from brain (BDNF) or Glial cell (GDNF). Tat-GDNF was injected intravenously to mice

Table II. Types of PTD-Fusion Protein and Usage

PTD	Protein	Cell Type/Animal	Treatment/Disease	Reference
Pep-1	SOD1	Hippocampus	Ischemic neurons	Cho *et al.*, 2008 (61)
Pep-1	Hsp27	Astrocyte/hippocampus	Brain ischemic insult	An *et al.*, 2008 (62)
Tat	Brain derived neurotrophic factor (BDNF)	Mice/primary hippocampal neurons	Neurodegenerative disease-Alzheimer's disease	Zhou *et al.*, 2008 (63)
Tat	Hsp70	Mouse/neuroblastoma cells	Parkinson's Disease	Nagel *et al.*, 2008 (64)
Antp HD	BIR3-RING (XIAP)	Hippocampal/rat	Brain Ischemia	Fan *et al.*, 2006 (65)
Tat	FNK	Isolated rat heart	Ischemia/reperfusion myocardial infarction	Arakawa *et al.*, 2007 (66)
VP22	GATA4	Mesenchymal stem cell/mouse	Ischemic cardiomyopathy	Bian et al., 2007 (60)
Tat	BH4/Bcl-x_L	Mouse	Sepsis	Hotchkiss *et al.*, 2006 (67)
Tat	FLIP	Lymphocytic jurkat/BJAB cell	Fas induced activation procaspase-8	Krautwald *et al.*, 2004 (68)
Tat	GDNF	Mice	Focal cerebral ischemia	Kilic *et al.*, 2003 (69)
Tat	Bcl-x_L	Mouse/cerebellar cell	Retrograde neuronal apoptosis	Dietz *et al.*, 2002 (70)
Tat	Bcl-x_L	Mice/murine brain mice/neuronal cell	Ischemic brain injury/neuronal apoptosis	Cao *et al.*, 2002 (58)
Tat	β-galactosidase (β-gal)	Isolated adult heart I/R	I/R cardiomyocyte	Gustafsson *et al.*, 2002 (71)
Tat	Bcl-x_L/PEA-15	Pancreatic β-cell line (islet)	Transplantable islet	Embury *et al.*, (2001) (59)

before and after ischemic/reperfusion and the number of caspase-3-immunoreactive and DNA-fragmented cells were significantly decreased while the number of viable neurons in the striatum increased.[69] This can be a powerful tool for acute treatment of stroke for its efficient delivery to the ischemic brain regions. Moreover, intravenous administration of Tat-BDNF fusion protein into neurons impaired in the Ab25-35 model mice recovered the brain function to the normal condition, which implies that the PTD-BDNF protects against amyloid peptide-induced learning and memory deficits.[63] BDNF-mediated neuroprotection by partially decreases the up-regulation of apoptotic proteins including phospho-Jun, cytochrome *c* and cleaved caspase 3.[74]

Recent research reported a potential therapeutic agent for the treatment of Parkinson's disease. Nagel *et al.* conjugated the heat-shock protein 70 (Hsp70) to Tat and tested *in vitro* as well as *in vivo* models of Parkinson's disease. Hsp70 as a molecular chaperone has the ability in reducing protein misfolding and aggregation, as well as in protecting the cells against oxidative stress and apoptotic stimuli. Tat-Hsp70 succesfully transduced neuroblastoma cells and provided protective effect in the primary mesenephalic dopaminergic (DA) neurons.[64] Besides Tat, Pep-1-SOD1 has been efficiently delivered into neurons plus having long-term neuroprotective effect against damage in the ischemic area where about 57% of hippocampal CA1 pyramidal neurons remained for 60 days after ischemia/reperfusion in hippocampus model.[61] An *et al.* conjugated Heat shock protein 27 (Hsp27) to PEP-1 and tested it against cell death and ischemic insults. The effect of Hsp27 was similar to Hsp70 as they belong to the same molecular chaperone family. In the culture medium of astrocyte and primary neuronal cells, PEP-1-Hsp27 showed protective effect against the cell death induced by oxidative stress.[62] After injecting Hsp27 fusion protein intraperitoneally into gerbils, the results showed that neuronal cell death in the CA1 region of hippocampus was markedly decreased in response to transient forebrain ischemia. Apart from that, Fan *et al.* showed that by using another apoptosis inhibitor, BIR3-RING (XIAP) conjugated to PTD-Antp HD showed promising results where BIR3-RING mainly inhibits the caspase-9 dependent apoptosis. The results demonstrated that PTD-BIR3-RING fusion protein could attenuate the cell death in oxygen glucose deprivation (OGD) hippocampal slices and decrease cell apoptosis in the rat transient middle cerebral artery ischemia (tMCAO) brains through inhibiting the caspase-3 apoptosis pathway.[65]

An artificial anti-cell death protein, FNK, which is genetically mutated form of Bcl-x_L has a better effect in anti-cell death activity against death stimuli.[75] Arakawa *et al.* injected Tat-FNK fusion protein intramuscularly into the anterior wall of left ventricle of ischemia/reperfusion model. Results showed that the infarct size reduced by 17% compared to the control at the highest concentration of 500 nmol/L of PTD-FNK.[66] Another caspase inhibitor and receptor-induced apoptosis inhibitor, β-galactosidase (β-gal) was fused to Tat and showed to protect H9c2 cells against apoptosis induced by hydrogen peroxide. Tat-β-gal was also perfused into isolated adult hearts before subjected to ischemia/reperfusion and shown to be cardioprotective.[71] Bian *et al.* transfected GATA4:VP22 into cardiac fibroblasts and transplanted them to Lewis rats, where the cardiac fibroblasts secreted GATA4:VP22 fusion protein by itself and consequently the

fusion protein entered directly to the targeted cells.[60] Six weeks after cardiac fibroblast transplantation (10 weeks after MI), GATA4:VP22 recipient animal demonstrated increased cardiac function and less negative remodeling. These systems may be applied to the myocardial infarction where the apoptosis is triggered when the cells undergo ischemia/reperfusion.

A novel autoantibody protein transduction domain, mAb 3E10 was developed as the single-chain fragment (Fv) of a murine anti-DNA autoantibody,[76] can deliver therapeutic peptides and proteins into cells and nuclei in the absence of cytotoxicity, differing it from the PTD mentioned above. This Fv fragment has been tested to fuse with a full length dystrophin as large as 427 kDa and was successfully delivered into the muscle cells.[77] Hansen *et al.* produced a fusion protein of Fv-Hsp70 and pretreated on the primary rat cortical neurons and COS-7 cells. The results showed the cytoprotective effect of Fv-Hsp70 in cells against oxidative stress from exposing to hydrogen peroxide.[78]

Single Chain Antibody (ScFv). Antibodies are invaluable *in vitro* and *in vivo* diagnostic tools due to their highly specific targeting characteristic. Single chain Fv fragments represents the minimal antigen-binding fragments of antibodies.[79] The advantages of small fragment to the whole antibody molecule include the reduced size while keeping the antigen binding site of molecule intact, the improved tissue penetration and the speed of penetration, rapid clearance from serum and reduced immunogenicity,[80] and facilitates the expression of the fragments and the fusions in bacteria. scFv can be served as building blocks to generate novel recombinant proteins such as bifunctional antibodies which consist of antibody fragments fused to another functional partner, for example an enzyme,[81,82] a toxin[83-85] or as a fusion to a lipoprotein[86] where the antibody fragment will be converted into a membrane-bound molecule where the antibody fragment will anchor on the bacterial membranes. Antibodies were widely used as the therapeutic agents in the treatment of cancer. Antibodies were conjugated with potent cytotoxic components like drugs,[87] or toxins.[88] However, there are limited studies on the antibody fused antiapoptotic protein.

ScFv-Antiapoptotic Protein. Huang *et al.* fused tumor necrosis factor (TNF-α) to the C-terminal of ScFv antibody which is specific for the human HER2/neu where the anti-HER2/neu ScFv-TNF-α retains both the TNF-α activity and the HER2/neu binding ability. The anti-HER2/neu ScFv-TNF-α induced potent HER2/neu signaling which activates the downstream mitogen-activated protein kinase (MAPK) and Akt pathways which will lead to the inhibition of apoptosis induced by actinomycin D.[89] This showed that the fusion protein has the potential to facilitate in wound healing especially of injured epithelia.

Mature T cells which undergo apoptosis may be an important pathophysiologic mechanism in disease like AIDS, cancer and autoimmunity.[90-92] Interleukin-2 (IL-2) is a growth factor for immune cells produced by activated T cells, has the protective effect on dexamethasone (DEX) or radiation-induced apoptosis in the immune cell-types like T cell clones,[93] antigen-specific T cells[94] and natural killer cells.[95] With the aim to protect T cells from dexamethasone-induced apoptosis, Kim *et al.* constructed a fusion protein (anti-CD3sFv-IL-2) in which anti-CD3 sFV will be targeted only to the CD3 epsilon moiety of T cell receptor. The results showed that the anti-CD3sFv-IL-2 fusion protein protected DEX treated T cells selectively and cell proliferation had been recovered. This specific targeting function of anti-CD3sFv can also reduce the undesired nonspecific antiapoptotic effect.

Drug Delivery System (DDS). Direct application of a protein by itself as therapeutic agent has some holdbacks due to its rapid elimination from the circulation due to renal filtration and enzymatic degradation, danger in triggering an immune response, and widespread distribution into non-targeted organs and tissues. These often result in non-economic and low efficient delivery and sometimes lead to non-specific toxicity. However, these drawbacks can be overcome with the protein-polymer conjugation where these conjugates will decrease the renal filtration by increasing the size of the protein by conjugating with the polymer.[96] Additionally, this conjugation can provide a protection to the protein against enzymatic degradation, as a consequence improve the protein stability and the longevity as well as lower the immunogenicity.

Polymer Drug Conjugates. At present, poly(ethylene glycol) (PEG) has been widely used in the field of drug delivery system.[97-100] This polymer is water soluble, has no electric charge, and is nonimmunogenic and nontoxic. Previous studies showed that PEG has a protective effect in early and long-term cold ischemia-reperfusion in renal medulla injury and the isolated perfused rat kidney model.[100] Bertuglia *et al.* demonstrated that PEG and newly developed organic nitrate forms of PEG (PEG-NO) can protect the microvascular perfusion in endothelial cells of hamster from oxidative stress and preserves shear stress mediated vasodilation in ischemia-reperfusion (I/R) injury.[101]

When the mitochondrial-dependent pathway is activated, apoptosome will be formed by cytochrome *c* activated Apaf-1, dATP and procaspase-9. From the family of N-alquilglycine inhibitors, peptoid1 was found to be the most effective apoptosome inhibitors in order to inhibit the apoptosis pathway.[102] The low membrane permeability and modest efficiency of the peptoid1 can be overcome by conjugation with poly-l-glutamic acid (PGA), forming more specific intracellular trafficking that coupled to an efficient lysosomotropic drug release on the cytosol to enhance the antiapoptotic activity of peptoid1.[103] PGA-peptoid1 was very stable in both

plasma and buffers but in the presence of lysosomal enzymes, the conjugates were degraded in a time-dependent manner reaching a plateu after 50 h. Maria and Enrique demonstrated that PGA-peptoid1 inhibits the activity of apoptosome which helps in silencing the caspases and lead to inhibition of caspase-dependent apoptosis pathway.[103]

Efficient Delivery of Nanoparticle Carrier. Colloidal carrier/nanoparticles carrier systems have drawn much attention lately as drug delivery carriers due to their high loading capacity as well as the protective characteristic towards the bioactive compounds against protein degradation.[104] Among the most popular drug carriers, liposomes are used mainly for the water soluble drug delivery whereas for the delivery of poorly soluble drugs are named micelles. Specific ligands (antibodies or certain sugar moieties) can be attached to the outer layer of the carrier to assist in the delivery or specific targeting into required sites in the cells.

PTDs conjugated with drug loaded nanoparticles (e.g. liposomes) assist the delivery of nanoparticles through cell membrane into cells. A study examined the kinetics of uptake of Tat- and penetratin-modified liposomes and found that the translocation efficiency was proportional to the number of peptide molecules attached to the liposomal surface and even five peptides are sufficient to enhance the liposomes delivery.[105] Liu *et al.* fabricated Tat-poly(ethylene glycol) (PEG)-β-cholesterol (Tat-PEG-β-Chol) and showed that the uptake of nanoparticles were able to cross the blood-brain barrier (BBB).[106] These nanoparticles can be used as a carrier for the delivery of brain infection drugs across blood-brain barrier. Apart from PTD-Tat, Antp was coupled to liposomes and the cellular uptake of PTD-modified liposomes was 15- to 25-fold increased compared to unmodified liposomes.[107]

A new liposome-based carrier that delivers its macromolecular cargo to the mitochondrial interior via membrane fusion (via macropinocytosis) was invented. A variety of diseases were caused by mitochondrial dysfunction, including Alzheimer's disease, Parkinson's disease, and diabetes mellitus.[108, 109] Thus, the first step towards restoration of a missing cellular function is to target the delivery of therapeutic products to nucleus or mitochondrion. Yamada *et al.* designed liposome particles, named MITO-porter, which carry octaarginine surface modifications to stimulate their entry into cells as intact vesicles via macropinocytosis. Upon release from the macropinosomes, MITO-Porter fused with mitochondrion via electrostatic binding with the mitochondrial membrane and then release of its cargo to the intramitochondrial compartment in living cells.[110]

Other than conjugating with PTD, liposomes can be further equipped with target specific ligands including receptors, antigens or antibodies (immunoliposomes). Coupling methods developed recently allow linkage of antibodies exposed on the liposome surface showing more efficient target binding.[111] Several recent *in vitro* and *in vivo* studies demonstrated that immunoliposomes bind specifically to their targets and deliver the encapsulated drugs into the cells.[111-114]

Summary

Conjugating antiapoptotic protein to PTD enhances the cellular uptake, interferes with the apoptotic agent and consequently inhibits apoptosis pathway. With the single chain fragment of antibody conjugated to the PTD fusion protein, it can specifically treat the targeted cells by applying with only a small amount of proteins. These antiapoptotic fusion proteins showed a promising future in the therapeutic treatment of diseases caused by dysregulated apoptosis. Finally, conjugating the fusion proteins with a polymer carrier will promote the stability and prolonged the effect of proteins in human bodies.

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