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Delivery of Intracellular-acting Biologics in Pro-Apoptotic Therapies

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Abstract

The recent elucidation of molecular regulators of apoptosis and their roles in cellular oncogenesis has motivated the development of biomacromolecular anticancer therapeutics that can activate intracellular apoptotic signaling pathways. Pharmaceutical scientists have employed a variety of classes of biologics toward this goal, including antisense oligodeoxynucleotides, small interfering RNA, proteins, antibodies, and peptides. However, stability in the in vivo environment, tumor-specific biodistribution, cell internalization, and localization to the intracellular microenvironment where the targeted molecule is localized pose significant challenges that limit the ability to directly apply intracellular-acting, pro-apoptotic biologics for therapeutic use. Thus, approaches to improve the pharmaceutical properties of therapeutic biomacromolecules are of great significance and have included chemically modifying the bioactive molecule itself or formulation with auxiliary compounds. Recently, promising advances in delivery of pro-apoptotic biomacromolecular agents have been made using tools such as peptide “stapling”, cell penetrating peptides, fusogenic peptides, liposomes, nanoparticles, smart polymers, and synergistic combinations of these components. This review will discuss the molecular mediators of cellular apoptosis, the respective mechanisms by which these mediators are dysregulated in cellular oncogenesis, the history and development of both nucleic-acid and amino-acid based drugs, and techniques to achieve intracellular delivery of these biologics. Finally, recent applications where pro-apoptotic functionality has been achieved through delivery of intracellular-acting biomacromolecular drugs will be highlighted.

Keywords

biomacromolecular drug; apoptosis; intracellular delivery; RNA interference; siRNA; peptide drug; endosome escape; delivery barriers

1. Introduction

Traditionally, cancer treatments have utilized relatively crude techniques such as surgical resection, chemotherapy, and radiation. These approaches can suffer from multiple shortcomings including incomplete eradication of the cancer, emergence of treatment-resistant cell phenotypes, and undesirable side-effects on healthy tissues. One of the basic paradigms of tumorigenesis is that cells that become defective or damaged are able to survive and grow in an abnormal, uncontrolled manner rather than succumbing to built-in programmed death pathways. An improved understanding of the signaling pathways

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responsible for tumor cell evasion of apoptosis combined with recent technological advances in the engineering of molecularly-targeted drugs will potentially revolutionize cancer therapies by enabling the rational design of specialized therapeutics that target cancer-specific phenomena. These advances could lead to cancer-specific, if not patient-specific, therapeutics tailored to directly combat the aberrant, tumorigenic molecules responsible for diverse neoplastic phenotypes.

The ability to more rationally design pro-apoptotic therapeutics for cancer is strongly dependent upon robust knockdown or inhibition of targeted molecules with great affinity and specificity. Synthetic small molecule drugs are the traditional approach for pharmacological inhibition, but synthesis and screening of prospective compounds is a long and tedious process and often yields products with suboptimal target specificity and/or pharmacokinetic profiles. In the normal, healthy cell, numerous levels of negative feedback exist for regulating molecular activities, including contributors at both post-transcriptional and post-translational levels. It may be ideal to utilize or mimic these “natural” mechanisms in the cell through delivery of therapeutics made from the same biologic building blocks (nucleic and amino acids) as the regulatory molecules endogenously present. For example, if exogenous doublestranded RNA with an antisense strand complementary to a target mRNA is delivered, it can commandeer the evolutionarily-conserved intracellular machinery in the cell to achieve sequence-specific gene silencing [1]. Alternatively, naturally-occurring protein antagonists or abbreviated peptide fragments containing their active sequences can be exogenously delivered to bolster the bioactive, intracellular pool of the antagonist, which may be functionally depleted (*i.e.*, not present or mutated) in certain cancers.

Biomimetic, nucleic acid- or amino acid-based drugs clearly have the potential to be therapeutically applied to alter intracellular apoptotic signaling pathways. However, delivery can be a challenge, especially for intracellularacting macromolecules, because they are generally not able to cross cellular membranes. This delivery barrier can have negative effects both on cellular internalization and on escape from intracellular vesicles (i.e., endosomes) that inhibit access to the microenvironment (*i.e.*, cytosol or mitochondria) where the molecular target is localized. For this reason, extracellular-acting molecules represent the largest sector of biologics currently used in the pharmaceutical industry. For example, biotechnological development of monoclonal antibody drugs is a strong and rapidly expanding field. Recent reviews indicate that there are at least 22 FDA-approved therapeutic monoclonal antibodies and hundreds more in the pipeline, with anticancer agents like Herceptin (trastuzumab), Rituxan (rituximab), and Avastin (bevacizumab) leading the way [2, 3]. This recent surge in biological drug development can be used as a precedent to indicate that establishing the basic infrastructure necessary for the manufacture of biologic drugs is feasible. Pro-apoptotic, RNA-based antisense drugs have also been extensively tested in clinical trials for multiple cancer types including melanomas, leukemias, lymphomas, gastroesophogeal carcinoma, and prostate cancer [4–9]. Although a distinct therapeutic advantage for use of antisense drugs has not been unequivocally proven at this point, the volume of trials in this area provide added optimism that intracellular-acting biologics can be feasibly developed for clinical use.

Overall, the accrual of new knowledge by cancer biologists to elucidate the molecular underpinnings of cancers has outpaced the rate at which pharmaceutical scientists and engineers have developed technologies that capitalize on this information clinically. Broadly-applicable platform technologies that confer the ability to efficiently deliver protein and RNA-based drugs intracellularly may be the key link to opening up a fertile new world of druggable targets that enable better clinical translation of new findings in cancer biology. Herein, the current molecular understanding of both the intrinsic (mitochondrial) and extrinsic (death receptor) apoptosis pathways is reviewed. Subsequently, delivery barriers of

intracellular-acting biomacromolecular apoptotic agents and a survey of the current pharmaceutical and biomaterial technologies that can be employed to overcome these barriers is discussed. Finally, a survey of recent pro-apoptotic therapeutic applications employing intracellular-acting biologic drugs, including the delivery techniques used and the molecular targets pursued, is presented.

2. Apoptosis

Apoptosis is a normal physiological process that occurs both in the homeostatic maintenance of adult tissues and during embryonic development. Apoptotic cells undergo a characteristic set of prominent morphological changes including alteration in cellular shape, nuclear and cytoplasmic condensation, and cellular fragmentation [10]. Eventually, apoptotic fragments of these cells are removed by phagocytes, and cytoplasmic leakage (i.e., necrosis) is avoided. Apoptosis can be triggered through both extrinsic and intrinsic pathways, where the extrinsic pathway is mediated by transmembrane death receptors, and the intrinsic pathway is triggered by a variety of factors [11] including environmental stress (e.g. hypoxia and reactive oxygen species), growth factor withdrawal [12], chemotherapy [13], and radiation [14]. These pathways converge at the level of activation of caspases, which are intracellular proteases that serve as the “executioners” of apoptosis [15, 16]. Under normal circumstances, caspase activation is primarily controlled by the B cell lymphoma 2 (Bcl-2) family of proteins, which provides upstream regulation of mitochondrial membrane potential [17, 18]. Importantly, mutations that lead to cancerous apoptotic resistance typically leave the intrinsic machinery intact, allowing it to be harnessed therapeutically [19].

2.1 Extrinsic Apoptosis Pathway

The extrinsic apoptotic pathway is regulated by members of the tumor necrosis factor (TNF) receptor superfamily known as death receptors (see Fig. 1). This group of transmembrane death receptors includes Fas (CD95/Apo-1) and the TNF-related apoptosis inducing ligand (TRAIL) receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) [20]. The Fas and TRAIL receptors are activated by the Fas ligand (FasL) and TRAIL, respectively, and these events result in the clustering of death receptors and the recruitment of the Fas-associated death domain (FADD) molecule [21]. In the case of TRAIL receptor activation, the recruited FADD molecule contains an effector region that catalyzes the caspase cascade by binding and activating caspase-8 and caspase-10 (known as the death-inducing signaling complex, or DISC). The activated Fas death receptor, on the other hand, recruits the death-associated protein (Daxx), which promotes apoptosis through activation of the apoptosis signal-regulating kinase-1 (ASK-1) and c-Jun N-terminal kinase (JNK) pathway [22]. Extracellular Inhibitors of the extrinsic pathway include membrane-bound decoy receptors 1–3 (DcR1, DcR2, DcR3), soluble Fas receptors (sFas), and soluble osteoprotegerin (OPG). Downstream from TRAIL receptor ligation, activation of caspase-8 is inhibited intracellularly by phosphoprotein enriched in astrocytes 15 (PEA-15) and cellular FLICE inhibitory protein (c-FLIP) [23–25]. Examples of potential therapeutic targets within the extrinsic pathway include activation of Fas and TRAIL death receptors as well as decreasing the expression of inhibitory molecules such as c-FLIP [26–28].

2.2 Intrinsic Apoptosis Pathway

A variety of cellular stresses activate the transcription factor p53, which is known as the tumor suppressor gene. p53 inhibits tumorigenesis by triggering apoptosis in stressed or damaged cells and operates through several proposed mechanisms including the transactivation of pro-apoptotic factors [29]. One of the important functions of p53 is its role in the Bcl-2 pathway. The members of the Bcl-2 family of proteins are key regulators of caspase activation and are considered to constitute the cell’s intrinsic “apoptotic switch” [17,

30]. There are two subsets of pro-apoptotic proteins in the Bcl-2 family and one subset of anti-apoptotic proteins, all of which contain one to four of the Bcl-2 homology (BH) domains. The first pro-apoptotic subset includes Bcl-2-associated X protein (Bax) and Bcl-2-antagonist/killer protein (Bak), both of which contain BH domains 1–3. Bax is normally free within the cytoplasm and translocates to the mitochondrial membrane following an apoptotic stimulus, whereas Bak permanently resides on the mitochondrial membrane [31]. Bak and Bax are generally considered to be redundant in function, and once activated, they each homo-oligomerize into multimers that insert into the mitochondrial membrane. These multimeric structures create “pores” that release cytochrome C, ATP, Smac, and Omi from the mitochondria to trigger a signaling cascade that results in caspase activation and apoptosis (see Fig. 1) [31, 32].

The other two subsets of Bcl-2 family members (Bcl-2-like and BH3-only proteins) are upstream mediators of apoptosis, and their interplay controls the activation of Bak and Bax. The Bcl-2-like subset includes members such as Bcl-2, Bcl-xL, and Mcl-1, and this group is anti-apoptotic in function. In general, these proteins contain all four BH domains, and the highly conserved BH1, BH2, and BH3 domains of Bcl-2-like proteins are thought to form a hydrophobic pocket that engages the BH3 domain of Bak/Bax [33, 34]. This interaction is the means by which the Bcl-2-like proteins neutralize the pro-apoptotic function of Bak/Bax. The ability of Bcl-2-like proteins to antagonize Bak/Bax and functionally inhibit apoptosis is believed to be determined by their relative activity compared to the third subset of the Bcl-2 family, the BH3-only proteins.

The members of the BH3-only subset, as the name implies, contain only the BH3 domain, and this group is represented by proteins such as Bim, Bid, Bad, Noxa, Puma, and several more. The BH3-only group promotes apoptosis by engaging the Bcl-2-like proteins in the same binding cleft that the Bcl-like proteins utilize to antagonistically bind to the BH3 domain of Bak and Bax [11]. Essentially, the BH3-only proteins compete with the BH3 domains of Bak/Bax for this binding pocket on the Bcl-2-like proteins, and by occupying this site, the BH3-only proteins inhibit Bcl-2-like inhibition of Bak/Bax and indirectly activate apoptosis [35, 36]. It is by this set of competing interactions that the relative balance of pro-apoptotic BH-3-only and pro-survival Bcl-2-like proteins is believed to control the apoptotic switch.

The discussed model for apoptotic control is known as the indirect activation model (i.e., BH3-only proteins indirectly activate Bak/Bax). It is important to note that a direct activatioBak and are inhibiten model has also been proposed where BH3-only proteins Bid and Bim directly activate Bax and d by anti-apoptotic Bcl-2, Bcl-X_L, Mcl-1, Bcl-w, and Bfl/A1. Bad, Bik, Noxa, Hrk, Bmf, and Puma are considered to be sensitizers that compete for the binding site of anti-apoptotic molecules in this model [37]. However, more recent studies have shown compelling evidence in favor of the indirect activation model, and the discussion herein will assume its accuracy [38, 39]. It is also important to note that the descriptions of the models provided in this review are somewhat simplified in that all members within each of the three Bcl-2 family subclasses are not identical in function. For example, cytosolic Bad binds to Bcl-2 and Bcl-X_L, which can indirectly activate Bak/Bax, enabling oligomerization, mitochondrial disruption, and apoptosis [40]. However, Bad interacts weakly with Mcl-1, and as a result, if Mcl-1 is predominantly present in the cell, apoptosis may still be resisted even in the presence of Bad [41]. In contrast, Noxa is known to bind only to Mcl-1 and A1 [38]. Although a discussion of the differences in the specific interactions mediated by each of the proteins within each of the Bcl-2 subsets is outside the scope of this review, these are important points to consider in drug design. For example, pro-apoptotic therapies that target specific Bcl-2 family members may not be effective if the

cells upregulate alternate Bcl-2-like members that are able to circumvent the drug activity and maintain pro-survival signaling.

There is also a family known as inhibitors of apoptosis proteins (IAPs) that operates downstream of Bak/Bax and activation of initiator caspases to prevent activation of the executioner caspases (caspase-3,-7, and -9) [42]. The IAP family has eight members including cellular-IAP1 (cIAP1), cIAP2, X-linked IAP (XIAP), Survivin, Bruce, and Livin, all of which contain the baculovirus IAP repeat (BIR) domain [43]. Negative regulators of the IAP family members also exist and examples include the pro-apoptotic molecules Smac and Omi, which are released upon depolarization of the mitochondrial membrane by Bak and Bax [44]. A diversity of tumors overexpress IAPs, with survivin in neuroblastoma and c-IAP1/2 in epithelial malignancies being notable examples, and this dysregulation contributes to their apoptotic resistance [45]. Current therapeutic targets within the IAP family include XIAP and Survivin inhibition or delivery of Smac or Omi to enhance chemotherapeutic effects [11, 46].

2.3 Oncogenes

"Oncogene" is a broad term for genes that are normally responsible for general cell maintenance and proliferation that have been mutated, resulting in tumorigenic phenotypes. Oncogenes can be derived from genes that normally encode a variety of proteins such as growth factors, growth factor receptors, transcription factors, and kinases. There are many examples of oncogenes, and this topic has been reviewed extensively elsewhere [47]. An important example of a cell survival pathway whose mutation is associated with oncogenesis is the epidermal growth factor receptor (EGFR) family of protein tyrosine kinases (PTKs). In humans, the EGFR family consists of the human epidermal receptors (HERs) and includes HER1, HER2, HER3, and HER4 [48, 49]. Activation of these protein tyrosine kinases results in downstream activation of pro-survival pathways such as the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and signal transducers and activators of transcription (STAT) pathways [50]. Akt can inhibit pro-apoptotic Bad, activate the pro-survival nuclear factor-κB (NF-κB), and also inhibit the pro-apoptotic transcription factor Forkhead [51]. NF-κβ activation induces the transcription of the key anti-apoptotic genes c-FLIP, Bcl-2, and Bcl-X_L, whereas Forkhead induces the transcription of the pro-apoptotic genes FasL and Bim [52, 53]. PTKs can also activate STAT-3 and STAT-5, which results in their translocation to the nucleus and transactivation of the anti-apoptotic genes Bcl-X_L, Mcl-1, and Survivin [54–56]. Cancer cells can become reliant on oncogenes for their sustained survival and growth, making oncongene inhibition an excellent target for induction of apoptosis. Current therapeutic strategies targeting the described oncogenic pro-survival pathway include using antibodies (e.g. cetuximab and panitumumab) or small molecule drugs (e.g. gefitinib and erlotinib) to inhibit EGFR binding [11], NF-κB activation (e.g. the proteasome inhibitor bortezomib) [57], or progression of the PI3K/Akt pathway [58]. The transcription factor c-myc represents another oncogene that is often dyregulated in cancerous cells [59], and therapeutic applications for the inhibition of this oncogene will also be discuess in more detail herein.

3. Biomacromolecular Drugs with Potential as Pro-apoptotic Therapeutics

3.1 RNA Interference

Controlled manipulation of gene expression using RNA interference (RNAi) has been rigorously pursued for almost two decades now, and thorough elucidation of this mechanism combined with recent breakthroughs in RNA delivery technologies have RNAi poised to make a tremendous impact on pro-apoptotic therapies. RNAi is an especially promising therapeutic approach for inhibition of anti-apoptotic Bcl-2-like proteins, oncogenes, or other

relevant targets because it allows for optimal therapeutic specificity and breadth (i.e., in theory, any desired gene target can be efficiently silenced). The initial discovery of RNAi came in 1990 when Napoli et al. observed an unexpected reduction in expression when delivering RNA in an attempt to overexpress chalcone synthase in Petunias [60]. Others elucidated and applied this finding by delivering antisense oligodeoxynucleotides (ODN), complementary sequences of DNA, which yielded modest reduction in gene expression in C. elegans [61]. In 1998, Fire *et al.* showed that intracellular-acting double stranded RNA (dsRNA) was more effective than either the sense or anti-sense strand alone [1]. In fact, dsRNA has been shown to be 100 to 1000 times more effective than ODNs due to a longer half-life and greater potency [62]. Over the next few years, researchers proved that endogenous RNAi, known as microRNA (miRNA), exist and that it serves as a natural, post-transcriptional controller of gene expression where cellular machinery selectively degrades complementary mRNA in an enzymatic manner [63]. The elucidation of similar machinery for RNAi in mammalian cells further heightened the interest in therapeutically harnessing these pathways [64].

Since these early findings, the mechanisms of ODN and that of miRNA, dsRNA, siRNA, and short hairpin RNA (shRNA) have been more clearly elucidated (see Fig. 2). Single-stranded antisense ODN are thought to function by multiple mechanisms including translational arrest due to steric blockage of ribosomes by ODN-mRNA Watson-Crick base pairing and also through RNase-H-mediated cleavage of both the ODN and mRNA strands [65]. Endogenous RNAi molecules in the form of microRNA (miRNA) enter the cytoplasm after transcription, or alternatively, functionally-similar dsRNA can be exogenously delivered. shRNA that more closely mimic the structure of endogenous miRNA have also been exogenously delivered [66]. In each of these cases, the RNase III family enzyme Dicer cleaves the miRNA/dsRNA/shRNA to produce guide RNA, more commonly known as small interfering RNA (siRNA). siRNA are double-stranded RNA 19–21 base pairs in length with 3’ nucleotide overhangs [67], these molecules can assemble into the RNA induced silencing complex (RISC), a nuclease complex that degrades complementary mRNA in a sequence specific, enzymatic manner [63]. RNAi efficiency has been further improved by delivering siRNA that directly enters the RISC rather than upstream dsRNA/shRNA that must be processed first by Dicer [68]. Because of the tremendous promise of siRNA to be used therapeutically (i.e., for silencing anti-apoptotic genes in cancer cells), most of the RNAi applications in this review will be focused on delivery of siRNA. However, to be used clinically, a number of significant delivery barriers must be overcome between initial application of the siRNA (i.e. intravenously or intratumorally) and its association with the RISC machinery in the cytoplasm of target cells.

3.2 Peptide-based Pro-apoptotic Strategies

The relative abundance of Bcl-2-like proteins and the BH3-only proteins is a major determinant in Bak/Bax activity and therefore, cellular homeostasis [69]. The disruption of this homeostasis caused by abnormal, excessive Bcl-2-like protein activity is thought to be a primary cause of both tumorigenesis and the ability of established tumors to resist conventional cancer therapies [70–73]. For example, dysregulation of apoptosis was first linked to neoplasia by Vaux *et. al* upon the elucidation of the function of Bcl-2 as a potential oncogene [74]. Since that initial report, Bcl-2 overexpression has been found to be a common hallmark of many cancers, especially lymphomas, and it is linked to decreased likelihood of cancer patient survival [71–73, 75–77]. As a result of the remarkable efforts of scientists to elucidate the apoptosis signaling pathway and to define the molecular causes for cellular oncogenesis, pharmaceutical scientists have been able to rationally design pro-apoptotic peptides and other amino acid-based drugs that target tumorigenesis at its molecular foundations. One promising strategy is application of peptides derived from the

BH3 domains of Bcl-2 family members. For example, minimal 16 amino acid sequences contained in the BH3 domains of pro-apoptotic Bcl2 family members are capable of mimicking the activity of full-length BH3-only proteins by occupying the binding site of Bcl2-like proteins and blocking their ability to repress Bak/Bax [78]. Therefore, synthesis and delivery of these peptides presents a logical approach for negating overexpression of Bcl-2-like proteins and triggering apoptosis in cancer cells (see Fig. 3). However, like RNA drugs, peptide drugs face a robust set of delivery challenges related to maintenance of stability, biodistribution to the tumor, and intracellular delivery to the microenvironment containing the pertinent molecular target.

4. Biomacromolecular Drug Delivery Barriers

Advances in the understanding of the molecular etiology of cellular oncogenesis have made it apparent that nucleic acids, peptides, and other biologics have great potential for specific manipulation of aberrant intracellular apoptosis signaling pathways to trigger death or chemosensitivity of cancer cells. However, the desired bioactivity of intracellular-acting pro-apoptotic biomacromolecular therapeutics can be limited by numerous delivery barriers including: proteolytic/nuclease degradation in the *in vivo* environment, opsonization leading to systemic clearance, inability to achieve specific targeting to the desired tissues/cells, non-specific binding and/or side-effects, inability to translocate the cellular membrane, inability to escape from the endo-lysosomal and exocytosis pathways, and lack of therapeutically-relevant concentrations of drug achieved within the intracellular microenvironment where the molecular target is located (Fig. 4) [79–82]. Stability of biologics is a primary concern considering the harsh environment encountered *in vivo*. The inherent intermediacy of RNA during gene expression predisposes it to having a relatively short half-life. For example, naked siRNA is degraded in minutes to one hour [83], and absorbance of serum proteins to drug carriers can opsonize them for removal from the body through the macrophages of the reticuloendothelial system (i.e., in the liver) [84, 85]. Furthermore, delivery to nontargeted, healthy tissues can lead to significant, non-specific cytotoxicity and negative side effects. Therefore, targeted delivery is often pursued to augment the effect of the drug and potentially avoid undesirable cell/tissue damage. The biologics discussed herein have therapeutic action in the cytoplasm, so cell membrane translocation is also essential. However, RNA- and amino acid-based drugs have relatively large molecular weight and hydrophilicity relative to small molecule drugs that can diffuse through lipid bilayer membranes. As a result, these drugs are internalized by endocytosis, which creates the added barrier of escaping from endo-lysosomal vesicles. Herein, currently available delivery technologies and recent pro-apoptotic applications are surveyed for both RNA- and amino acid-based therapeutics.

5. Overcoming Barriers to Delivery of Amino Acid- and Nucleic Acid-Based Therapeutics

Advances in pharmaceutical technology and an increased understanding of the pharmacokinetics of biomacromolecular drugs have led to the development of a variety of tools to address systemic and intracellular delivery barriers. Pharmaceutical techniques for efficient intracellular delivery of peptide/protein therapeutics and siRNA include electroporation, fusion with cell-penetrating peptides (CPPs) and/or fusogenic peptides, chemical modifications that convey pharmaceutical properties onto the bioactive molecule itself (i.e. peptide “stapling”), formulation into liposomes/nanoparticles, and conjugation to “smart” polymers, among others. The specific approach utilized is typically selected based upon the pathological application and class of drug being delivered. For example, while neither peptide nor siRNA therapeutics can traverse cell membranes by simple diffusion, there are additional RNA-specific properties that pose unique delivery challenges. One

distinguishing characteristic of RNA is that it is a polyanionic material due to the negative charges from the phosphate groups in the RNA backbone. Since anionic macromolecules are not very efficiently internalized by cells, approaches for shielding the neagative charges on nucleic acids (i.e. by loading into liposomes or formulation into cationic polyplexes) is an important consideration that is typically less relevant for peptide delivery.

5.1 Electroporation, Iontophoresis, and Sonophoresis

There are a variety of relatively simplistic techniques useful for in vitro transfection or for in vivo application to superficial pathologies where the primary goal is getting past the skin barrier. For example, electroporation is a technique where high voltage pulses are applied to the target tissue (usually skin) triggering a voltage drop across the cell membrane of locally affected cells. This stimulus disrupts the lipid structure and forms aqueous pathways in the membrane that allow biomacromolecules to diffuse across [86]. Despite the lack of a clear mechanistic understanding of the permeabilization process, it is widely accepted that electric fields above 200 mV form nanopores in the cell membrane [87–89]. Electroporation has been used to deliver siRNA, proteins, and antibodies to various cell types. For example, optimized electroporation of deoxycytidine kinase (dCK) siRNA into acute T-cell lymphoblastic CEM cells resulted in 70–80% suppression of dCK mRNA and its enzyme activity [90]. Similarly, novel electroporation microchips have been shown to achieve transfection of the GFP plasmid into HEK-293 cells with transfection efficiency as high as 90% [91]. There are also numerous protein delivery applications that have been pursued. Electroporation of the enzyme β-galactosidase into murine melanoma cells has been achieved at efficiencies of 20% [87], and electroporation has also been used to deliver exogenous antigens into the cytoplasm of T-hybridoma cells in order to preferentially activate class I MHC processing [92]. In addition, it was found that electroporation delivered detectable levels of monoclonal antibodies against G1-specific cyclin D1into 80% of CV1 and MCF7 cells, and intracellular delivery of the antibody successfully inhibited mitosis in these cells [93]. Although electroporation can potentially affect cell viability, optimized electroporation protocols have resulted in techniques that sufficiently permeabilize cell membranes (*i.e.* >90% of cells) for therapeutic translocation while maintaining 80–90% cell viability [94, 95].

Another technique similar to electroporation is iontophoresis, which involves application of a constant current to move charged proteins or siRNA (i.e., through the epidermis) [96, 97]. Due to the ion-driven nature of this approach, delivery efficiency correlates to the charge of the drug utilized, and example applications of this technique include transdermal delivery of the insulin protein [97–99] and siRNA delivery for ocular gene therapy [100]. Application of ultrasound has also been explored as a means of peptide delivery (*i.e.* low-frequency sonophoresis) [101]. Sonophoresis is thought to disrupt lipid structure in various tissues, and tissue-specificity is achievable by varying the frequency of ultrasound waves applied. This approach has primarily been studied for applications involving transdermal drug delivery. Although iontophoresis and sonophoresis can be used to effectively translate through the skin’s stratum corneum, they do not necessarily enable cellular internalization. Thus, electroporation is the most promising technique within this category based on potential for intracellular delivery. However, loss of cell viability and the inability to pursue *in vivo* applications where less superficial tissues are targeted represent key limitations of electroporation.

5.2 Photochemical Internalization and Laser Irradiation

Other techniques explored for biomacromolecular drug delivery include photochemical internalization and tissue permeation using laser irradiation. Photochemical internalization is a technique that relies on the localization of amphiphilic photosensitizing agents into the

endosomal membrane. Upon exposure to a light source, these agents generate highly-reactive singlet oxygen species that damage and permeabilize the endosome [102]. The use of photochemical internalization as a tool for intracellular delivery was originally proposed by Berg *et. al.* who utilized lysosomes as photochemical targets [103]. Subsequently, Berg and authors used photochemical internalization for the cytosolic delivery of the protein toxin gelonin, as well as for the site-specific, photochemically enhanced delivery of the anti-neoplastic glycopeptide bleomycin [104, 105]. Photochemical internalization has also been utilized for prolonging siRNA-mediated gene silencing [106]. Despite the intracellular biomacromolecular drug delivery potential of photochemical internalization, current applications are limited by the lack of supporting technologies (i.e., fiber-optic probes) that can be employed to activate photochemical agents in deeper tissues in vivo.

Another technique with applicability for the delivery of pro-apoptotic peptides and siRNA is laser-irradiation induced tissue permeation, specifically with an erbium:YAG laser [107–109]. Use of laser irradiation has been found to increase antigen-induced antibody production following skin vaccination and enhanced transdermal delivery of a number of other therapeutic agents including vitamin C, 5-fluoruracil, and 5-aminolevulinic acid [107–109]. Additionally, skin pre-treatment with a low-fluence erbium:YAG laser has been shown to increase transdermal siRNA delivery up to 10-fold compared to untreated groups [110]. The mechanism underlying the laser irradiation delivery method is thought to involve the disruption of intercellular connections (*i.e.* gap junctions) allowing for enhanced drug permeation through the skin barrier [107]. However, like electroporation and iontophoresis, laser irradiation is used to enhance the transdermal transport of drugs, and it is limited by the inability to overcome intracellular delivery barriers.

5.3 Cell-Penetrating Peptides

CPPs, also known as protein transduction domains (PTDs), were originally developed to mimic viruses that are able to translocate their own genetic material across cell and endosomal membranes. The 1988 discovery that the transactivating transcriptional factor (TAT) of HIV-1 can penetrate cell membranes [111, 112], followed by the discovery of antennapedia (a.k.a. penetratin) which is derived from *Drosophila* [113], rapidly stimulated the use of CPPs for intracellular biomacromolecular drug delivery. The finding that the antennapedia peptide was rich in positively charged arginine residues subsequently motivated the development of synthetic, arginine-rich CPPs (AR_CPPs, or oligoarginines) [114]. A multitude of other CPPs have since been discovered from naturally occurring sequences or synthetically designed, including transportan, VE-cadherin derived peptide (pVEC), the herpes simplex virus type I derived CPP VP22, diatos peptide vectors (Vectocell®), various oligoarginines, and many other novel sequences [88, 114–120]. A representative list of some of the most widely studied CPPs is given in Table 1.

The mechanism of cellular internalization of CPPs is thought to vary depending on the peptide sequence, the architecture of the drug formulation, and the cargo being delivered [121]. Cell entry mechanisms are hypothesized to include membrane fusion via binding of CPPs to cell surface proteoglycans, the endocytosis pathway (caveolindependent, clathrin-dependent, or caveolin- and clathrin-dependent), and macropinocytosis [121–124]. If the CPP and its cargo are taken up through endocytosis or macropinocytosis and a mechanism to escape these intracellular vesicles (inherent or through modification) is not present, the drug may be either degraded in the lysosome, remain sequestered in the early or late endosome, or undergo trafficking for exocytosis [121, 125]. Thus, a combinatorial therapeutic approach is commonly employed where the cell-penetrating properties of a CPP are coupled with the endosomolytic properties of fusogenic proteins/peptides or pH–responsive “smart” polymers [125–129]. Additional consideration must also be given to ensure that the CPP does not result in non-specific side effects. For example, Ward and co-

authors reported unexpected effects of CPPs on kinase inhibition [130]. Furthermore, some CPPs have also been shown to have cytotoxic effects depending on the synthetic route and CPP formulation used (e.g. some *retroinverso* cell penetrating peptides have been shown to result in severe cytotoxicity mediated through nonspecific side effects) [131]. Other potential disadvantages of CPPs include poor serum and protease stability and their indiscriminate cell entry, which could be problematic for systemic delivery applications where specific cells (i.e., in a tumor) are targeted. Herein, CPP delivery of pro-apoptotic biomacromolecules will be the focus. However, CPP conjugation to small molecule chemotherapeutics (i.e., doxorubicin and camptothecin) has also been assessed and found to either meet or exceed current standards achievable through delivery of the "free" drug [132–134].

5.4 Fusogenic Peptides

In order to avoid sequestration or degradation within the endo-lysosomal pathway, peptides with membrane fusogenic activity can be employed. These peptides are generally amphipathic in structure and transition into a more lipophilic state at slightly acidic pH (*i.e.*, in the microenvironment of endo-lysosomes), allowing them to partition into and disrupt lipid bilayer membranes [135]. Initially, the understanding of the structure and function of fusogenic peptides depended upon studies on the viral and cellular fusion proteins found in nature. These fusion proteins are generally either glycoproteins contained in viral envelopes or proteins involved with fusion protein attachment receptors (i.e., soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE)) [136]. In the case of the viral envelop, this interaction involves insertion of an α-helical peptide domain into the cell membrane catalyzing fusion of the viral envelope with the cellular or endosomal membrane. One of the most widely utilized fusogenic peptides is the N-terminal sequence of the Influenza hemagglutinin subunit HA-2 and its derivatives [137–140]. A multitude of other fusogenic proteins have also been discovered or created, including synthetic hemagglutinin derivatives, model amphipathic peptides (MAPs), β-amyloid trans-membrane protein derivatives, membrane-disruptive peptides found in various toxins and venoms, and a family of fusion-associated small transmembrane (FAST) proteins (*i.e.* the p10 avian reovirus, the p14 reptilian reovirus, and the p15 baboon reovirus) [121, 135–148]. A representative list of fusogenic peptides is given in Table 2. Note that penetratin, transportan, and TAT are classified as both fusogenic peptides as well as CPPs due to their fusion based mechanism of cell entry at physiological pH. In contrast, other fusogenic peptides listed typically rely on a stimulus, such as lowered pH, in order to catalyze membrane fusion, and, therefore, they are best used toward endo-lysosomal escape as opposed to cellular uptake.

5.5 Liposomes / Nanoparticles

Colloidal systems, including liposomes, micelles, and nanoparticles, are widely used for drug delivery because they can carry large drug quantities, physically shield drug cargo from enzymatic activity, and provide simple routes for modification with other molecules that can be used to finely tune the pharmaceutical properties [149]. For example, multiple functional components can be incorporated through loading (i.e., drug) into the interior of the nanoparticle or surface modification with moieties such as cell-targeting molecules, CPPs, fusogenic peptides, pHresponsive polymers for endosomal escape, and hydrophilic polymers (*i.e.* PEGylation for increased stealth within the systemic environment). Modification with the CPP TAT has been one of the more widely utilized approaches, while other colloidal drug carrier modifications include alternate CPPs such as R8 and IRQ, fusogenic peptides such as GALA and penetratin, and a variety of molecules including nuclear localization signals (NLS) and extracellular receptor-targeting ligands such as folic acid [121, 126, 128, 149–154]. Mitochondria-targeted vehicles are one especially promising, recent development

in colloidal drug carriers pertinent to the delivery of pro-apoptotic peptides. Since mitochondria are central to controlling cellular apoptosis, targeted drug delivery to these organelles may enable development of more potent pro-apoptotic therapies. One successful liposome-based approach to mitochondrial drug delivery incorporated the mitochondriotropic lipid triphenylphosphonium (TPP), mitochondrial targeting signal (MTS) peptides, CPPs, or synergistic combinations of these components [155]. However, significant challenges to mitochondrial drug delivery still exist, especially for applications that require control of intra mitochondrial trafficking. This combinatorial approach, however, provides a good example of the complex optimization possible through modulation of the composition and stoichiometric ratios of the different components of multifunctional particulate carriers.

One of the most common methods for nucleic acid transfection is formulation with lipids [156–158]. Lipid agents can form vesicles with a lipid bilayer membrane surrounding an aqueous interior where hydrophilic drugs can be encapsulated, or cationic lipids can be used to form lipoplexes with anionic nucleic acids. Lipids can either fuse to the cell membrane or enter the cell by endocytosis, eventually releasing their cargo into the cytoplasm [159, 160]. Information on liposome preparation, physicochemical properties, and applications can be found in more detail in a review of liposomal drug delivery systems by Samad *et al*. [160]. Lipids are commonly combined with other components to incorporate added functionality. For example, Morrissey *et al* used a cationic and fusogenic lipid (SNALP) delivery vesicle coated with a PEG-lipid layer to provide a neutral, hydrophilic exterior [161]. As a result of the multi-functional potential of liposomes and nanoparticles, they have also become promising candidates for the treatment of drug resistant cancers. For example, Huang and authors have developed multifunctional liposomes with a removable PEG shield that are capable of highly efficient tumor biodistribution and intracellular co-delivery of siRNA and doxorubicin to combat tumor cells with multi-drug resistant (MDR) phenotypes [162, 163]. In another analogous design, a guanidinium-containing liposome incorporated multiple components including a P-glycoprotein drug efflux pump inhibitor to combat drug resistance, anisamide for cancer cell targeting, and doxorubicin as a chemotherapeutic agent. The guanidinium compounds have been shown to generate reactive oxygen species (ROS) such as hydroxyl free radicals (•OH) [164], and these ROS radicals are hypothesized to act as second messengers that reduce MDR phenotype through activation of c-Jun N-terminal kinase (JNK) and c-Jun [165, 166].

A variety of inorganic nanoparticles and polyplex- or micelle-forming polymers have also been pursued as biomacromolecular delivery vehicles. In some applications, especially with inorganic nanoparticles, technological advances have been pursued that combine both image contrast and therapeutic functionalities. For example, siRNA-loaded Au nanoparticles (AuNPs) coated with cationic polymers have been reported to increase RNA half-life 6-fold when compared to naked RNA *in vitro* [167]. Quantum dot nanoparticles, renowned for their optical properties, have also been functionalized to successfully deliver siRNA and have tremendous potential to serve as “theranostic” platforms [168, 169]. Several natural organic polymers have also been used to make polyplex nanoparticles for siRNA delivery including sugar containing polymers such as atelocollagen [170, 171] and chitosan [172]. One important consideration in fabrication of nanoparticles is control of particle size and polydispersity. For example, control of nanoparticle size and aggregation for polyplexes is typically controlled by adjusting the nitrogen to phosphate charge ratio (N/P ratio). Kong *et al.* showed that the hydrodynamic diameter of nanoparticles consisting of poly[2-(dimethylamino) ethyl methacrylate] PDMAEMA/siRNA binary polyplexes could be varied from 102 nm to 58 nm simply by increasing the N/P ratio from 1 to 3 [173]. However, for polyplexes, further consideration must be given to the possibility that siRNA bioactivity could be hindered by stable (nonreversible) complexation with a polycationic carrier. One

method to address this issue is to utilize bioreducible disulfide linkages to connect siRNA to their carriers rather than using electrostatic complexation, a technique that has demonstrated significant improvements in transfection efficiency with no apparent cytotoxic effects [174, 175].

5.6 "Smart" pH-responsive Polymers

The ability to escape endo-lysosomal pathways in order to avoid degradation or exocytotic recycling out of the cell represents one of the most important carrier functionalities for biomacromolecular drug cytosolic delivery. "Smart", pH-responsive synthetic polymers with membrane-disruptive behavior that mimics the functionality of fusogenic peptides have been intensely studied for this purpose. This class of delivery systems can take the form of soluble polymer-drug conjugates, polyplexes, and micelles, and, thus, it overlaps partially with the more general "nanoparticle" category. These polymer therapeutics are typically designed to be activated by the decreasing pH gradient experienced during trafficking from the extracellular environment (i.e. pH ~7.4) into the intracellular endolysosomal pathway (i.e., 6.8 > pH > 5). "Smart" polymers can be finely-tuned to respond in this physiologicallyrelevant pH range to trigger endo-lysosomal vesicle disruption, and this functionality can be harnessed to deliver therapeutic biomacromolecules to the cytosol.

Two primary subclasses of synthetic, pH-responsive polymers exist (anionic and cationic), and they produce endo-lysosomal escape through very different mechanisms (see Fig. 5 for structures of representative anionic and cationic pH-responsive polymers). The anionic polymers, for example, become more hydrophobic in acidic environments, triggering adoption of a less solvated, compact globule conformation that partitions into and disrupts lipid bilayers [176–179]. Some of the most thoroughly studied pH-responsive polymers are poly(alkylacrylic acids), a family of anionic polymers that was primarily pioneered by the Tirrell laboratory's work on poly(ethylacrylic acid) (PEAA) [176, 178]. Examples of other anionic, pH-sensitive polymers that have since been applied for intracellular delivery include poly(styrene-alt-maleic anhydride) (PSMA), poly(propylacrylic acid) (PPAA), and various copolymers containing PPAA and PSMA [127, 129, 180–183]. Murthy et. al. developed a related class of "encrypted" polymers that also have lipophilic activity that disrupts endo-lysosomal membranes. In the encrypted delivery system, PEG polymers attached via acid-labile acetal linkages "shield" a hydrophobic, endosomolytic polymer backbone until being shed upon exposure to acidic pH [183, 184]. We and others have successfully applied members of the anionic smart polymer subclass toward intracellular delivery of polymer-peptide and polymerprotein soluble conjugates as well as for siRNA delivery using particulate polyplex- and micelle-based formulations [125, 127, 185, 186].

A variety of cationic endosomolytic polymers have also been developed, and this alternate subclass of "smart" polymers employs a colloid osmotic mechanism described by Behr et. al. as the "proton sponge" effect [129, 176, 177, 180, 187]. Following internalization of extracellular cargo, early endosomal compartments are normally acidified by the action of an ATPase-driven proton pump in their membrane. The proton sponge effect is triggered by weakly basic cationic polymers that "absorb" the protons pumped into these vesicles, which buffers against the acidification of the endosomal microenvironment. This buffering triggers continued, abnormal import of protons and their counter-ions, establishing an osmotic imbalance that triggers water influx, vesicle swelling, and endosomal disruption [75]. Examples from this class of pH-responsive polymers include poly(L-lysine), linear and branched poly(ethylenimine) (PEI), poly(amidoamine) (PAMAM) dendrimers, poly(β-amino esters) (PBAE), and histidine and/or imidazole containing copolymers [188–194]. Due to the inherent electrostatic interaction between positive and negative macromolecules, it is logical to utilize cationic polymers to condense nucleic-acid based drugs. The electrostatic packaging approach also protects the nucleic acids by providing resistance to

nucleases present in vivo. However, the cationic nature of these polymers also predisposes them to indiscrimate cellular uptake, instability due to competing interactions with charged serum or extracellular matrix proteins, and cytotoxicity. All of these are significant barriers to clinical use of polycations, and as a result, recent developments have been aimed at overcoming these shortcomings. For example, for the “gold standard” polycation PEI, cytotoxicity and serum instability can both be improved simply by using lower molecular weight PEIs (5–48 kDa) [195–197]. Alternatively, partial modification of PEI amines with other molecules, such as poly(ethylene glycol), imidazole, peptides, or receptor-targeting moieties has also been shown to reduce nonspecific cellular uptake and cytotoxicity [198–202]. Polymer biodegradability can also be a significant advantage as exemplified by PBAE carriers, which decompose into cytocompatible, low molecular weight degradation products and are significantly less toxic than PEI and poly(L-lysine) [203, 204]. A representative list of cationic polymer-mediated siRNA delivery applications is given in Table 3.

5.7 PEGylation

Drugs designed for intravenous delivery must be equipped to navigate a complex variety of potential interactions with erythrocytes, serum proteins in the blood, and extracellular matrix proteins in the target tissue prior to cell uptake. For example, opsonization with serum proteins can “mark” drug carriers for removal from the body through the reticuloendothelial system, and anionic matrix proteins such as glycosaminoglycans can destroy the integrity of electrostatic polyplexes [84, 85, 205]. PEGylation is the primary method used to increase longevity in the circulation and make drugs more inert in order to minimize nonspecific cell uptake, drug aggregation, immunogenicity, and toxicity [206–211]. PEGylation is well-accepted to increase *in vivo* blood circulation half-life [212], but it can also reduce therapeutic efficacy by interfering with cellular uptake and endosomal escape [209]. For example, Hatakeyama and coworkers found gene silencing achievable using a lipid-based nano-device carrying interfering RNA to vary from 70% to 5%, with level of knockdown activity being inversely proportional to amount of PEGylation [213]. This observation suggests that the degree of PEGylation and PEG chain lengths should be optimized to balance inhibition of undesirable drug interactions with maintenance of bioactivity. For example, Gao *et al.* tested a series of liposomes with varied degrees of PEGylation for siRNA delivery, and liposomes containing 2.5% PEG showed the best HER1 gene silencing activity [214]. Other studies have indicated that a degree of substitution in the range of 10 to 30 using a PEG chain length of 2000 Da to be the most effective approach to stealth liposome formulation [202]. Wang and authors demonstrated PEGylated nanoparticles to reduce cytotoxicity relative to traditional formulations with PEI or cationic liposomes, although this was at the expense of significantly reduced cellular uptake. However, the loss of uptake could be compensated for by inclusion of the targeting peptide Bombesin, and this also enabled better cell-type specificity by limiting internalization of the carrier to receptormediated uptake [211]. In another study, Huang *et al.* recently found that the “shedability” of PEG from the surface of liposomes played an important role in improving tumor biodistribution and activity. In these studies, an unprecedented 70–80% of the injected drug dose accumulated within xenograft tumors, and effective silencing of oncogenes was achieved at impressively low (*i.e.*, <1mg/kg) siRNA doses [208, 215, 216]. Finally, another means to minimize negative effects of PEGylation is to incorporate cleavable peptide linkages between the base carrier and the PEG chains such that the PEG layer is cleaved off by enzyme (i.e., matrix metalloproteinase (MMP)) activity present in the target tissue [213]. Ultimately, PEGylation provides a method to increase systemic half-life and avoid non-specific interactions with blood components, but targeting molecules enabling receptor-mediated uptake or removal of the PEG chains may be necessary for optimal therapeutic activity.

5.8 Drug Targeting

Ligands or antibodies specific to internalized extracellular receptors that are expressed specifically on targeted cell types can be used to achieve drug selectivity in the body. For example, the peptide (EPPT), which targets the tumor-specific, underglycosylated MUC-1 (uMUC-1) antigen, has been used to deliver siRNA to HBV cells [161]. Other examples include anisamide, a ligand of sigma receptor that is overexpressed in many human malignant tumor cells [162] and folic acid (FA), a ligand whose receptor is highly expressed in many epithelial cancers [217–219]. Another common cancer cell target is the transferrin receptor (TrfR), a glycoprotein involved in iron homeostasis and cell growth that is overexpressed on numerous tumor cells [220]. In fact, the amount of transferrin receptors on malignant cells has been shown to be as much as 100-fold greater than on normal cells [221, 222]. As a result, transferrin [223–225] and TrfR antibodies [226–228] have been used in a number of targeted drug delivery applications. These examples provide a sampling of the receptor-mediated targeting used to enhance drug uptake by cancer cells.

5.9 Chemical Modifications of Biomacromolecular Drugs

5.9.a. siRNA Design and Modification—Many of the characteristics of idealized siRNA sequences were initially identified by Elbashir and authors. For example they discovered that duplexes made of 21 base pairs with 3’ overhangs 2 nucleotides in length are optimal, and they also determined that mismatches between the antisense siRNA and mRNA in the middle of the siRNA can abolish activity, while the 3’ nucleotide has little effect on gene silencing [64, 68, 229]. Reynolds and co-authors more recently completed a systematic, mass screening of siRNA sequences for 2 genes and identified low Guanine/Cytidine content, a low internal stability at the 3’ end of the sense strand, and lack of inverted repeats as desirable siRNA characteristics in addition to uncovering sense strand base preferences at specific sites in the sequence [230]. Since the primary focus here is on the carriers utilized for siRNA delivery, the reader is referred to reviews by Aigner for a more thorough discussion on siRNA sequence optimization [231, 232]. One common approach to silencing a new gene is to acquire several siRNA sequences that satisfy these design criteria and that target different loci on the mRNA. The sequences can be either pooled together or screened individually to identify an optimal sequence for further study. Off-target gene silencing and nonspecific immune responses mediated through toll-like receptors can also be triggered by suboptimal siRNA sequences, and instances have occurred where nonspecific effects have been misinterpreted as siRNA-driven phenotype modification resulting from silencing of a target gene [233]. However, siRNA therapeutic potential remains strong as chemical modifications and new rules for sequence identification are progressing in-step with the iterative improvements in delivery approaches. In preclinical studies, testing for immune activation and verifying phenotypes independently using different siRNA sequences against the gene of interest are desirable standards of practice [234].

5.9.b. Peptide Stapling—Generally, peptides have poor *in vivo* stability, cellular uptake, and overall pharmacokinetics, but recent synthetic advances have led to the development of peptidic therapeutics endowed with these properties. With this approach, peptide intracellular delivery barriers can be overcome through the physicochemical properties of the peptide therapeutic itself as opposed to gaining these functionalities through conjugation to a peptide/polymer or loading into a liposome or nanoparticle. The modification of peptide therapeutics to improve stability and potency was initiated by the advent of *retroinverso* peptides, or peptides in which each naturally occurring L-amino acid is replaced with the D-enantiomer and the peptide bonds are reversed [235]. In addition to *retroinverso* peptides, chemical cross-linking has also been used to stabilize secondary structure of peptides, and this method of stabilizing peptides containing α-helices increases stability of the secondary

structure and peptide potency [236]. The crosslinking chemistry as well as the chemoselectivity of the reaction can significantly affect the pharmacological and structural properties of the peptide and continue to be optimized for various therapeutics [237]. Classical approaches to stabilization of α-helices make use of covalent bonding between the *i* and *i+4* or *i* and *i+7* side chain groups and are comprised of lactam, disulfide, or metal-mediated linkages [237–240].

Early peptide cross-linking approaches have since evolved into the development of stapled peptides, or peptides in which non-natural, olefin-conjugated amino acids are used to incorporate controllable cross-links. The link between olefin groups consists of an all hydrocarbon 'staple' between successive turns (*i.e.*, *i* and *i+4*) of the α-helical structure and is formed through a ring-closing ruthenium-catalyzed olefin metathesis reaction [241–243]. Blackwell and Grubbs first described stapled peptides [244], and later, the Verdine laboratory further optimized the metathesis reaction and the overall stapled peptide design [243]. Use of an all hydrocarbon 'staple' can improve structural stability, potency, protease resistance, and even cell permeability [241]. The ability of stapled peptides to achieve cellular entry is hypothesized to be a consequence of their stabilized structure and has been shown to involve an energy-dependent endocytotic uptake mechanism [245]. Stapled peptides are evolving into an indispensable tool for the intracellular delivery of pro-apoptotic sequences, and significant clinical promise has been shown for the stapled peptides known as stabilized α-helix of BCL-2 domains, or SAHBs [241].

6. Pro-apoptotic RNAi Therapeutic Applications

Cancer biologists have provided a relatively thorough understanding of the aberrantly expressed genes that grant cancerous cells their hallmark ability to evade apoptosis, possess self-sufficiency in growth signals, display high proliferative potential, promote angiogenesis, and metastatically invade other tissues [246]. RNAi has been pursued for a diversity of gene targets responsible for these traits, both those where silencing directly induces apoptosis and those that sensitize cells to secondary therapeutics. Initial RNAi applications in cancer therapy were primarily ODN approaches, and 21 example gene targets that have been pursued with antisense directed at inducing apoptosis in breast cancer were reviewed in a 2002 report [247]. However, due to the subsequent elucidation of the enzymatic mechanism and higher potency of double-stranded siRNA, the field has predominantly shifted from ODN toward siRNA approaches for cancer therapies [65]. The more recent approaches have utilized siRNA to manipulate targets in the Bcl-2 family and IAPs, in addition to other targets related to cell signaling, viral oncogenes, cell cycle, metabolism, and nutrient transport [248]. Robustly testing for therapeutic effects mediated by siRNA is contingent upon successful siRNA intracellular delivery and target gene silencing. Supplementary Materials Table 1 surveys a sampling of published siRNA delivery approaches and the relative level of gene knockdown that has been achieved using different transfection techniques. Herein, we specifically highlight siRNA applications in pro-apoptotic therapies, and because clinical utilization of siRNA is the ultimate goal, we attempt to differentiate between reports where apoptosis-related targets where tested *in vitro* (Table 4) versus the more translational studies done *in vivo* (Table 5).

6.1 Bcl-2 Family Proteins

The most direct and aggressively pursued pro-apoptotic RNAi method has been silencing of Bcl-2-like proteins Bcl-2 and Bcl-xl [249]. Knockdown of these Bcl-2-like subfamily members allows the indirect activation and homo-oligomerization of Bax/Bak, the critical pro-apoptotic triggers of the mitochondrial apoptosis pathway (see Fig. 1). RNAi of Bcl-2 has been proven to increase apoptosis and arrest growth of HeLa and several other cancer cell lines, and the apoptotic effect can be amplified with co-delivery of chemotherapeutics

[250, 251]. Poeck *et al.* introduced 5'-triphosphate modifications onto Bcl-2 siRNA, with the triphosphate chemistry being incorporated to activate Retinoic acid-inducible gene 1 (RIG-1) in innate immune cells. This triggered interferon expression, creating an immune response that acted synergistically with Bcl-2 silencing, and the combined effects produced 50% apoptosis of melanoma cells and a reduction in tumor size in mice [252]. Silencing of intracellular Bcl-2 has also been utilized to increase sensitivity to activators of the extrinsic apoptosis pathway (i.e., TRAIL) in melanomas, but Bcl-2 knockdown was found to be a less effective sensitizer relative to knockdown of the IAPs Survivin and XIAP [253].

6.2 IAPs

Apoptotic resistance in various cancers can, in part, be attributed to the overexpression of specific IAPs. For example, Survivin and Livin are overexpressed in neuroblastoma, and c-IAP1 and c-IAP2 are overexpressed in epithelial malignancies [45, 254]. As a result, silencing of IAPs has also been pursued as a route to reduce cancer cell resistance to apoptosis [255]. Survivin is released from the mitochondria during apoptotic signaling to negatively regulate caspase activation [256], and its silencing in HeLa cells has been shown to induce apoptosis [257]. However, in other cell lines like human sarcoma, the apoptotic effect was limited to only 10% of the cells when no secondary treatment was applied [258]. In a more recent in vivo study, Survivin siRNA (4 mg/dose) complexed with DharmaFECT was injected intratumorally into mice twice per week over a 3 week period. This approach resulted in a 40% reduction in human lung adenocarcinoma tumor weights, and co-delivery of Survivin siRNA with cisplatin produced a stronger antitumor response than either drug alone [259]. Livin is a newly discovered IAP that inhibits caspases 3, 7, 9, and cytochrome c, making it a logical choice for pro-apoptotic RNAi therapy. Knockdown of Livin created an apoptotic effect in HeLa cells [257] but only limited response in other cell types. XIAP is another IAP that inhibits caspases 3, 7, and 9, and thus, it is also a viable target for RNAi-mediated apoptosis. Dan and co-authors found that approximately 80% apoptosis was induced when XIAP was inhibited in ovarian cancer cells [260]. Furthermore, in vivo transfection of XIAP siRNA using intratumoral injection and electrotransfection pulsing decreased tumor cell proliferation by 75% relative to control groups in a 50-day tumor study on MCF-7 breast cancer xenografts [261].

6.3 Oncogenes

Normal cells regulate the transcription factor c-myc tightly, but in cancerous cells, mutations in c-myc deregulate its expression, triggering uncontrolled cellular proliferation [59]. In fact, mutation of c-myc is a hallmark of some cancers (i.e., Burkitt's lymphoma), and in some cases it is essential for cancer cell survival, making it a promising potential drug target [262]. For example, *in vitro* silencing of c-myc (80% knockdown) in MCF-7 cells has been found to reduce their growth rate both *in vitro* and *in vivo* (in cells transfected prior to their inoculation into the mouse), and a 40% induction of apoptosis was measured upon serum withdrawal in c-myc deficient cells versus only 6% in controls [263]. Systemic delivery of siRNA through electrostatic condensation onto protamine-functionalized targeting antibodies has been used for delivery of pooled siRNA sequences against c-myc, MDM2, and VEGF, which was shown to reduce the size of subcutaneous B16 melanoma tumors by more than 50% relative to control groups at a 9-day endpoint [264]. Huang and co-authors have thoroughly assessed c-myc knockdown in mouse models using lipid-based carriers, and they have found that c-myc silencing effectively induces apoptosis and reduces tumor growth [262, 265, 266].

Although c-myc has been a primary oncogene drug target, inhibition of other onconges has also been pursued therapeutically. HER2 is an example of an oncogene that is often dysregulated in many types of cancers and causes excessive cell growth. This oncogene is

particularly relevant in breast cancer, and siRNA targeting HER2 has been shown to arrest growth and trigger apoptosis in vitro in several cell types, including HER2-expressing MCF-7 breast cancer cells [267]. Polo-like kinase 1 (PLK-1) is another gene involved in regulation of cell cycle progression. Many cancer cells overexpress PLK-1, and it is hypothesized that this aberrant expression can cause excessive cell growth and inhibition of p53-induced activation of apoptosis [268]. PLK-1 overexpression promotes chromosome instability and overrides mitotic checkpoints leading to immature cell division [269]. Silencing the expression of PLK-1 in nasopharyngeal carcinoma (C666-1) cells with systemic delivery of siRNA has been found to result in significant apoptosis and decreased tumor growth [270]. In addition to nasopharyngeal carcinoma, dramatically increased apoptosis has been shown to result from PLK-1 siRNA silencing in breast cancer cells, HeLa cells, colon cancer cells, and lung cancer cells, with as high as 50% apoptotic induction reported in some cell lines [271]. Furthermore Benoit, Henry, and authors showed that an approximately 50% reduction of PLK-1 gene expression in NCI/ADR-RES and OVCAR8 ovarian cancer cells lines sensitized these cells to doxorubicin cytotoxicity [272]. This study, among others discussed below, achieved powerful, synergistic effects by co-delivering siRNA and small molecule chemotherapeutic drugs.

6.4 Gene Targets for Sensitization to Radiation and Chemotherapy

Most of the studies discussed up to this point demonstrate the potential of siRNA to directly induce apoptosis through silencing of Bcl-2 proteins, IAPs, and oncogenes. However, many of these studies have indicated that application of siRNA alone is often not enough to create a robust pro-apoptotic effect, and, thus, multi-component approaches may be optimal. One common approach is to simultaneously deliver chemotherapeutics and RNAi agents designed to silence genes known to cause cancer chemoresistance [273]. This approach is especially relevant for cancer patients whose previously treated, residual tumors develop multidrug resistance (MDR). MDR is a genetic or acquired trait of cancer cells that exhibit resistance against multiple, structurally or mechanistically unrelated anticancer drugs. MDR cancer cells are characterized by poor uptake, increased excretion, and elevated metabolism of drugs (see Fig. 6). Drugs that are influenced by reduced uptake are mostly water soluble drugs that are internalized by routes similar to nutrients, and these include antifolate methotrexate, nucleotide analogues (5-fluorouracil and 8-azaguanine), and cisplatin [274, 275]. Drug resistance caused by increased drug efflux from the cell affects drugs that are ejected through cell membrane transporters, and these drugs include anthracyclines (doxorubicin (Dox) and daunorubicin), vinca alkaloids (vinblastine and vincristine), RNA transcription inhibitor actinomycin-D, and the microtubule-stabilizing drug paclitaxel [276]. In many cases, MDR cells simultaneously acquire mechanisms that impede intracellular drug accumulation and also undergo oncogenic alterations that diminish their susceptibility to the drugs.

The "classical" MDR phenotype results from decreased intracellular drug accumulation due to the drug efflux activity of the adenosine triphosphate binding cassette (ABC)-transporter MDR1/P-glycoprotein (MDR1/P-gp, ABCB1) encoded by the human MDR1 gene. Overexpression of P-gp frequently contributes to MDR phenotype because it transports a broad spectrum of drugs including Dox, vinblastine, and paclitaxel out of the cell. Development of P-gp inhibitors selectively targeting MDR cancer cells to decrease drug efflux may sensitize these cells to chemotherapy and improve patient outcomes [277]. To this end, more than a 10-fold decrease in the LC_{50} of amrubicinol was recently observed in an amrubicinol-resistant small-cell lung cancer cell line (PC-6/AMR-OH) treated with P-gp siRNA (~70% knockdown achieved) relative to controls with basal P-gp levels [278]. Another recent study tested P-gp silencing as an adjunct to Bortezomib, which is a selective inhibitor of the proteasomal pathway. Despite its potency in chemoresistant myeloma

patients, some patients become refractory to prolonged treatment [279, 280]. Rumpold *et al.* demonstrated that sensitivity to Bortezomib could be restored by knockdown of P-gp with siRNA [281]. In this study, siRNA silencing of P-gp was found to improve the EC_{50} of Bortezomib from 25 mg/mL to 4.5 mg/mL and the EC_{50} of MLN273 (another proteasomal inhibitor) from 253 ng/mL to 9.8 ng/mL in MDR myeloma cells. Minimizing the active dose, in addition to cell-specific targeting (if achievable), are of paramount importance in P-gp silencing approaches because P-gp serves as a vital cellular transport mechanism in normal cells, and its nonspecific inhibition can lead to negative side effects.

Ral-interacting protein (RLIP76) is another multifunctional membrane transporter, involved in stress-defense and apoptosis resistance. RLIP76 transports glutathione-conjugate (GS-E) compounds, thus controlling the intracellular concentration of pro-apoptotic, oxidized lipid byproducts and also chemotherapeutic agents. Depletion or suppression of RLIP76 with antibodies or siRNA confers increased chemosensitivity (e.g., to 2–3 fold higher cell death from 4-hydroxynonenal in small cell lung cancer) [282–284]. Furthermore, recent studies have shown that inhibition or depletion of RLIP76 by antibodies, siRNA, or antisense can lead to drastic and sustained regression of lung, kidney, melanoma, colon, and prostate cancer xenografts with no observed recurrence of tumors and no evident toxicity [282, 285–289].

The oncogene c-Myc has also been proposed to contribute to MDR efflux pump expression [290, 291], and for this reason inhibition of c-Myc has been hypothesized to enhance tumor sensitivity to chemotherapeutics. Dox is known to be especially susceptible to MDR efflux pump activity [292], and Huang and authors have attempted to address this problem through co-delivery of Dox with siRNA against c-Myc. In this study, it was demonstrated that delivery of c-Myc siRNA to mice with MDR ovarian cancer xenografts down-regulated efflux pump expression and promoted nearly 2 times greater tumor uptake of Dox relative to controls not receiving c-Myc siRNA [162]. However, other evidence suggests that c-Myc knockdown and chemotherapy treatments may be more effective if done sequentially rather than simultaneously. For example, silencing of c-Myc in medulloblastoma cells was found to slow cell growth. However, c-Myc knockdown also decreased cell sensitivity to cisplatin, etoposide, and ionizing radiation and reduced apoptosis by 24–56%, presumably due to the key role c-Myc plays in apoptotic signaling in these cells [293].

Cyclin D1 proto-oncogene is another potentially relevant anticancer gene target because it is an important regulator of G1 to S-phase transition during cell division, and it also serves as a cofactor for several transcription factors. Cyclin D1 is overexpressed in many human pancreatic cancers, a cancer type with an especially poor patient prognosis due to shortcomings in early detection and the fact that pancreatic tumors are commonly resistant to chemotherapeutic agents [294, 295]. Thus, improvement of pancreatic cancer therapies is an area of significant clinical need, and siRNA knockdown of Cyclin D1 may have promise in this area. For example, siRNA silencing of Cyclin D1 in pancreatic tumor cells has been shown to enhance cisplatin- and fluoropyrimidine-mediated growth inhibition. In these studies, cisplatin- and fluoropyrimidine-mediated apoptosis was enhanced by approximately 3-fold in Cyclin D1-overexpressing pancreatic tumor cells delivered Cyclin D1 siRNA relative to cells treated with a control siRNA [294]. Several studies have also demonstrated that the anti-apoptotic, tumorigenic activity of D-type cyclins (D1, D2, and D3) may be dependent on cooperative interaction with other growth promoting genes such as c-Myc and Ras [296, 297]. For example, co-expression of c-Myc and cyclin D3 promotes lymphoid cell resistance to dexamethasone-induced apoptosis, and overexpression of cyclin D1 inhibits drug-induced apoptosis in rat embryo fibroblasts ectopically expressing c-myc [298]. Furthermore, cyclin D1 positively correlates with high basal and cisplatin-induced activity of NF-κB, which contributes to chemoresistance and cell survival through inducing

expression of anti-apoptotic Bcl-2 family of proteins [299, 300]. Finally, a recent investigation by Biliran et al. revealed a relationship between c-Myc and cyclin D1 that affects cell apoptosis and drug sensitivity [301] (see Fig. 7). This exemplifies the complex interrelationships that occur between oncogenes during tumorigenesis and provides a number of viable gene targets whose silencing may act synergistically with other cancer therapies.

In addition to serving as an adjunct to small molecule drugs, gene silencing has also been utilized to sensitize cancer cells to radiation therapy. For example, DNA-dependent protein kinase (DNA-PK) is hypothesized to make cells resistant to radiation therapy, and An and co-authors tested the effect of DNA-PK silencing in HeLa cells. The authors demonstrated a 7-fold decrease in cancer cell number 6 days after a 2 Gy dose of irradiation in HeLa cells delivered siRNA targeting the catalytic motif of DNA-PK relative to control cells receiving irradiation only. Interestingly, the hypothesized mechanism for this effect was a concomitant repression (~80% decrease) of c-Myc expression in cells where DNA-PK was silenced [302]. Thus, the DNA-PK gene could be an attractive anticancer gene target for sensitization of cancer cells to radiation therapy whose inhibition may lead to co-repression of other harmful oncogenes.

7. Pro-apoptotic Peptide Therapeutic Applications

The intracellular delivery of peptides that mimic BH3-only protein activity is one of the most extensively studied amino acid-based pro-apoptotic strategies [125, 241, 242, 245, 303–306]. Peptide therapeutics occupy a small but promising niche among other classes of pro-apoptotic anticancer drugs such as therapeutic antibodies and small molecule Bcl-2 antagonists [307–309]. Among the pro-apoptotic peptide sequences derived from BH3 domains, stapled BH3 peptides (SAHBs) may have the most ideal pharmacological properties because of their improved stability of the α-helical structure, protease resistance, cell permeability, and increased affinity to the multi-domain, BH3 binding “pockets” on Bcl-2-like proteins [245]. However, BH3 peptide mimics continue to be developed and screened in new formulations in an effort to optimize their therapeutic potential. The utilization of BH3 peptides has also stimulated the discovery of small molecule drugs, including ABT-737 and ABT-263, which have similar functionality to BH3-only peptides [303, 306, 310]. Peptide mimics of BH3-only proteins have been derived from several of the BH3-only proteins (*i.e.* Bim, Bid, Bad, Noxa, Puma, *etc.*) and also from the BH3 domains of Bak and Bax. Peptides derived from BH3 domains of different parent proteins have different specificities, and, clinically, this may be important for the design of cancer- or patient-specific therapeutic regimes. For example, Shangary and co-authors demonstrated that agents based on the Bax BH3 domain may be best aimed at cancers overexpressing Bcl-2, while peptides derived from the BH3 domain of Bad may be more useful for tumors overexpressing Bcl-xL [311]. Generally, BH3 peptides have shown promise as pro-apoptotic agents, and herein we will survey the results of tests where they have been delivered using electroporation, CPPs, stapled peptides, liposomes/nanoparticles, and pH-responsive smart polymers.

Pro-apoptotic peptides derived from the BH3 domains of Bak and Bax have been tested with electroporation, and it was found that at 48 hours after peptide delivery, cell viability was reduced by 40% in prostate tumor cells [312]. BH3 peptides have also been conjugated with CPPs to improve intracellular delivery. For example, peptides derived from the Bak BH3 domain have been fused with the CPP penetratin, and this approach resulted in ~80% decrease in HeLa cell viability relative to the unmodified BH3 peptide control [304]. Likewise, a truncated Bax-derived BH3 peptide fused with TAT (*i.e.* pTAT-p3Bax) chemosensitized NRP-154 prostate epithelial cells and enhanced induction of apoptosis by

thapsigargin (a non-competitive inhibitor of sarco-endoplasmic reticulum Ca^{2+} ATPase) [313].

In addition to the use of CPPs to enhance intracellular delivery of pro-apoptotic therapeutics, cell-specific targeting peptides/ligands can be utilized to achieve tumor selectivity. For example, Minko and authors delivered apoptotic BH3 peptide drugs targeted with the Luteinizing-hormone-releasing hormone (LHRH) peptide, a molecule whose receptor is overexpressed on numerous cancer cell types. In this study, the LHRH-BH3 peptide produced a significant loss in cell viability (peptide IC_{50} 3.97 ± 0.33 ng/mL) in A2780 human ovarian carcinoma cells, while no significant toxicity was caused by the BH3 peptide without the LHRH sequence [314]. CPP-conjugates have also been created that incorporate mechanisms for tissue-targeted delivery. In one example of this approach, the antineoplastic agent chlorambucil was conjugated to a chimeric peptide containing the cyclic, breast-tumor targeting peptide cCPGPEGAGC (PEGA) the CPP pVEC. In breast cancer MCF-7 cells, the IC_{50} for chlorambucil was 128 μM, while chlorambucil conjugated to the PEGA-*p*VEC had an approximately 4-fold lower IC_{50} of 30 μM [133]. In a prostate cancer application, Li *et al.* developed a novel fusion peptide (BSD352) that consisted of the CPP TAT, a sequence from the BH3-only protein Puma (TAT-$BH3_{Puma}$), an anti-vascular endothelial growth factor peptide (SP5.2), and an anti-basic fibroblast growth factor peptide (DG2). It was found that the components of the fusion peptide acted synergistically to inhibit the growth of androgen-sensitive human prostate adenocarcinoma (LNCaP) xenograft tumors in a mouse model, and tumor volume was decreased from ~1500 mm^3 to ~300 mm^3 in treated mice relative to PBS controls. Mechanistically, the fusion peptide was found to elevate levels of Bax, enhance cytochrome c release, and induce caspase-9 cleavage to induce tumor cell apoptosis while simultaneously inhibiting angiogenesis and endothelial cell proliferation [315]. However, despite observing a dose-dependent, synergistic apoptotic effect in LNCaP cells, minimal levels of apoptosis were observed in PC3 and RWPE-1 cell lines. Finally, tissue specific delivery has also been sought using photochemical internalization. In this realm, Shamay et al. have tested a light-activated, caged CPP fused with a pro-apoptotic peptide $(D)(KLAKLAK)_2$, a peptide that was designed by Ellerby and coworkers [316]. It was found that upon 10 minutes of light exposure, 80% of the therapeutic was internalized, producing a 90% loss of viability in PC3 prostate cancer cells [317].

Stapled peptides have also been used in several pro-apoptotic peptide delivery applications and have shown potential as potent in vivo therapeutics. Walensky et al. tested a BH3 stapled peptide, or SAHB, derived from the BH3 domain of the BID protein in a murine RS4;11 leukemia xenograft model. Their results demonstrated that daily treatment with the stapled peptide significantly prolonged survival and, by day 5, regression of leukemia cells from the spleen and kidney was detectable, while leukemic expansion was apparent at these sites in the control group [245]. In another leukemia model, Moellering and authors used a stapled peptide termed SAHM1 to inhibit the NOTCH transcription factor in a murine model of T-cell acute lymphoblastic leukemia (T-ALL). It was found that daily treatment with the SAHM1 therapeutic resulted in dose-dependent tumor regression and a 5-fold increase in caspase 3/7 activities in treated cells [318]. Other preliminary studies that have not yet reached in vivo testing are exploring new SAHBs, and in one recent application, Stewart and authors reported promising in vitro results from a Mcl-1-derived SAHB Mcl-1 inhibitor [319].

Liposomes have also been employed as pro-apoptotic delivery vehicles with promising results in animal tumor models. Ko *et al.* reported that cationic liposomes loaded with pro-apoptotic peptide $(D)(KLAKLAK)_2$ and Bcl-2 antisense oligodeoxynucleotide G3139 inhibited B16(F10) melanoma tumor growth in a mouse model by over 50% (reduction in tumor size from ~1000 mm^3 to <500 mm^3) and that the inhibitory effects correlated with

increased caspase activity [154]. Additionally, Xu and authors studied the therapeutic efficacy of a liposomal carrier loaded with caspase-6 and anti-human epidermal growth factor receptor (HER2) antibody in both HER2 positive (*i.e.* SK-BR-3 human breast cancer and SKOV-3 human ovarian cancer) and HER2 negative (i.e HeLa human utero-cervical carcinoma and BT325 human glioma) cancer cell lines [307, 308]. Time-dependent cell death was observed in HER2 positive cells, whereas no significant effect was observed in HER2 negative cells. In a murine SK-BR-3 tumor xenograft model, liposomes loaded with the anti-HER2 antibody resulted in a >50% decrease in tumor growth and prolonged survival when compared to the liposome alone. In a parallel study, Wang and authors utilized the same antibody-loaded liposome approach to prolong survival and significantly inhibit tumor metastasis in a murine model of human osteosarcoma [308].

"Smart" pH-responsive polymers have also been investigated for the intracellular delivery of pro-apoptotic amino acid-based biologic drugs. For example, we have developed a multifunctional, endosomolytic polymer that enhanced delivery and pro-apoptotic effects of a CPP-BH3 peptide [79]. This delivery system is based on poly(N-(2-hydroxypropylmethacrylamide))-b-poly(propylacrylic acid-co-butyl methacrylate-co-dimethylaminoethyl methacrylate) (pHPMA-b-p(PAA-co-BMA-co-DMAEMA) containing a pyridyl disulfide polymer chain end functionality incorporated through a reversible addition fragmentation chain transfer (RAFT)-based synthetic procedure. This reversible pyridyl disulfide conjugation site was utilized so that the peptide could be released from its carrier in the reducing environment of the cytoplasm. The pHPMA block was designed to confer long circulation times, and the propylacrylic acid-containing pH-responsive terpolymer block was incorporated to mediate pH-activated endosomal escape. Peptide conjugates with pHPMA-b-p(PAA-co-BMA-co-DMAEMA increased HeLa cervical carcinoma cell apoptotic activity over free peptide and resulted in 50% tumor cell death in cultures after 6 h of treatment [79]. More recently, we have also utilized pH-responsive homopolymers of propylacrylic acid to deliver protein antigens for anticancer vaccines. In these studies, ovalbumin was incorporated as a model protein antigen to test the ability of these polymer-based vaccines to stimulate class I MHC presentation and antigen-specific cytotoxic T-cell activation. These activities require cytosolic delivery and processing of the antigen, which was augmented in this case using the endosomolytic polymer carrier. In an in vivo study using the EG.7-OVA mouse tumor protection model, PPAA ovalbumin conjugates produced a functionally significant prophylactic effect. PPAA-based protein vaccine delivery facilitated an 8-fold increase in antigen-specific CTL production and an approximately 3.5-fold increase in the length of tumor-free survival relative to mice delivered vehicle controls [185].

8. Conclusions

The discovery of new pro-apoptotic drug targets, the development of new delivery techniques for intracellularacting biologics, and the identification of novel cell-targeting strategies are providing researchers with the tools needed to produce more effective anti-cancer therapies. As demonstrated through numerous in vivo preclinical tests, delivery of pro-apoptotic siRNA, proteins, peptides, and antibodies is being optimized through continually evolving approaches to overcome delivery barriers. Among these approaches, stapled peptides and stealth liposomes have great potential, especially with respect to the improved stability and specificity of stapled peptides and the capability of recent liposome formulations to deliver as much as 80% of systemically injected payloads into a tumor. These promising technologies enable scientists to unlock a new universe of intracellular targets that are druggable by biomacromolecules. The optimal targets for these drugs tend to be dependent on the type of cancer, and future treatments may be tailored to patient-specific cancer phenotypes. While these approaches will potentially be utilized to develop new

stand-alone therapeutics, the most promise to this point has been demonstrated through synergistic combinations of biomacromolecular drugs and currently available chemotherapeutics. Thus, future trends may include further development and optimization of technologies that can successfully navigate the array of both extra- and intracellular barriers to achieve co-delivery of biologic and small molecule drugs. Rapid progress in both the fundamental understanding of apoptosis and in the engineering of improved delivery systems suggests that clinical successes utilizing intracellular-acting pro-apoptotic biomacromolecular drugs are forthcoming.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

STAT	Signal tranducers and activators of transcription
RNAi	RNA interference
shRNA	Short-hairpin RNA
siRNA	Small interfering RNA
uMUC-1	Underglycosylated MUC-1
FA	Folic acid
TrfR	Transferrin receptor
CPP	Cell penetrating peptides
MAP	Mitogen-activated protein
MAPK14	p38 MAP kinase
PCD	Cystamine bisacrylamide-diaminohexane
PCM	Primary cardiomyocyte
MEND	Multifunctional envelope type nano device comprised of lipid/protein transduction domain peptide
TUNEL	TdT dUTP nick end labeling
Dox	Doxorubicin
CDP	Cyclodextrin-containing polycations
AuNPs	Au nanoparticles
PAMAM	Poly(amidoamine)
EHCO	N-(1-aminoethyl)iminobis[N-(oleicyl-cysteinyl-Histinyl-1-aminoethyl)propionamide
PBAE	Poly(B-amino esters)
RGD	Arg-Gly-Asp

MDR	Multi drug resistance
ROS	Reactive oxygen species
JNK	c-Jun N-terminal kinase
BN	Bombesin
StA	Stearic acid
RIG-1	Retinoic acid-inducible gene 1
plk-1	Polo-like kinase 1
RRM2	Ribonucletodide reductase
ABC	ATP-binding cassette
DNA-PK	DNA-dependent protein kinase
RLIP76	RaI-interacting protein
GS-E	Glutathione-conjugate
P-gp	P-glycoprotein
AMR	Amrubicinol
FDA	Food and Drug Administration
PTDs	Protein transduction domains
TAT	Trans-activating transcriptional factor
pVEC	VE-cadherin derived peptide
FAST	Fusion-associated small transmembrane
MAPs	Model amphipathic peptides
NLS	Nuclear localization signal
TPP	Triphenylphosphonium
MTS	Mitochondrial targeting signal
PSMA	Poly(styrene-alt-maleic anhydride
PPAA	Poly(propylacryic acid)
PEI	Polyethylenimine
PAMAM	Poly(amidoamine
SAHBs	Stabilized α-helix of BCL-2 domains
Bim	Bcl-2-interacting mediator of cell death
Bid	BH3-interacting-domain death agonist
Bad	Bcl-2-associated death promoter
Bmf	Bcl-2-modifying factor
Puma	P53-upregulated modulator of apoptosis
Bik	Bcl-2-interacting killer
Hrk	Harakiri
LHRH	Leutinizing-hormone-releasing hormone

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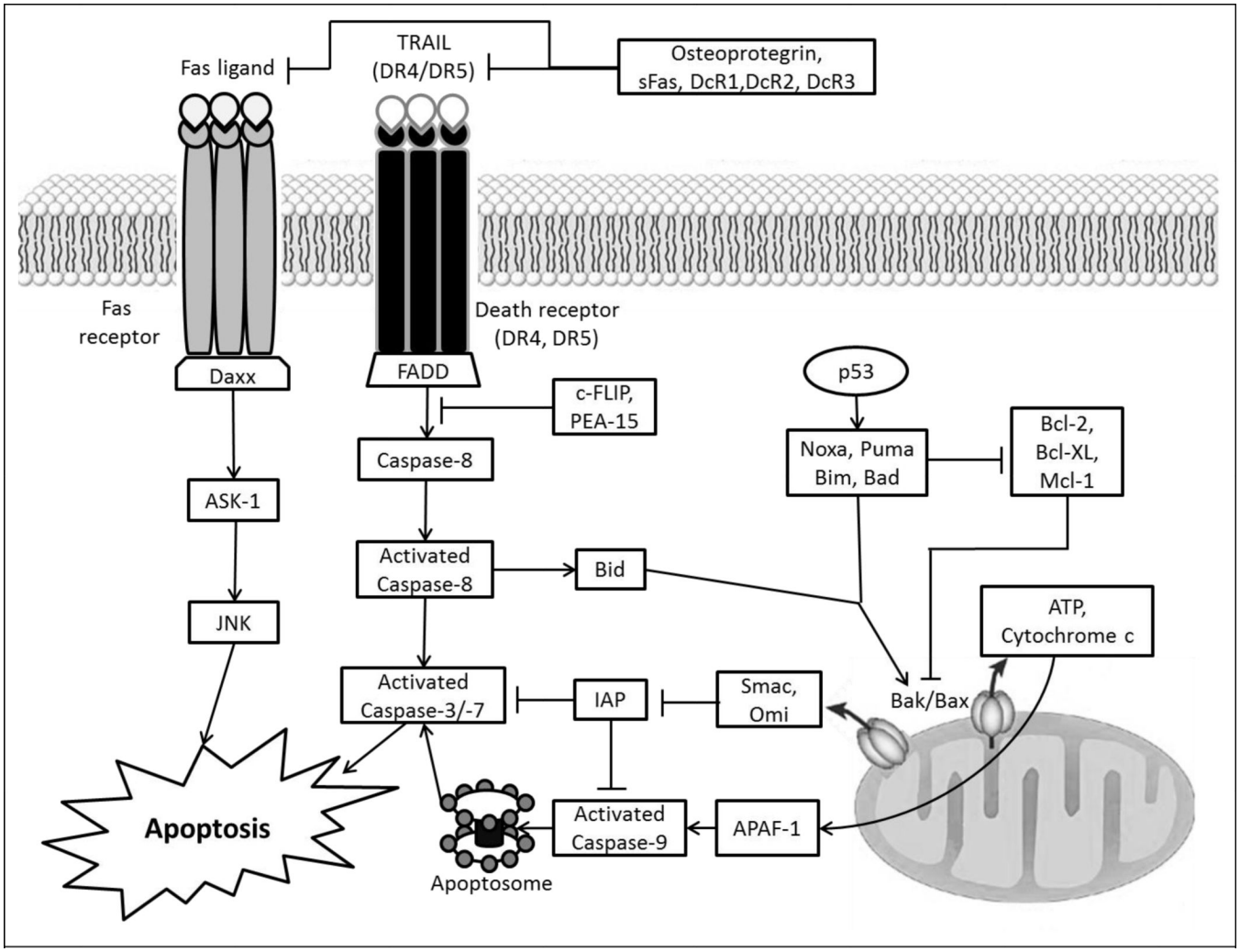

Figure 1. Schematic summarizing the cellular intrinsic and extrinsic apoptosis pathways

The extrinsic pathway is activated by receptors such as Fas, DR4, and DR5 that, when bound by their ligands, associate with cytosolic adapter death domains, i.e., FADD and Daxx. The FADD complex eventually activates executioner caspases (−3 and −7) while (Fas-specific) Daxx leads to caspase-independent apoptosis mediated downstream through JNK. Osteoprotegerin, sFas, DcR1, DcR2, and DcR3 inhibit the ligation of apoptotic death receptors, and PEA-15 and c-FLIP are relevant inhibitors of caspase-8 activation via the FADD pathway. The activation of the intrinsic (or mitochondrial) pathway is predominantly controlled by the Bcl-2 family proteins. BH3 only members (*i.e.* Bim, Bad, Bid, Noxa, Puma) either activate Bak and Bax directly (direct activation model) or indirectly (indirect activation model) through inhibition of the anti-apoptotic Bcl-2-like proteins, *i.e.* Bcl-2, Bcl-X_L, and Mcl-1. Activated Bak/Bax homo-oligomerize and form mitochondrial pores that release pro-apoptotic factors such as ATP, cytochrome c, Smac, and Omi into the cytosol. Cytochrome c, ATP, APAF-1, and activated caspase-9 form the apoptosome, which can activate executioner caspases (*i.e.*, −3, and −7). Smac and Omi further promote apoptosis by preventing IAPs, which are caspase inhibitors. Schematic modified from [11, 19].

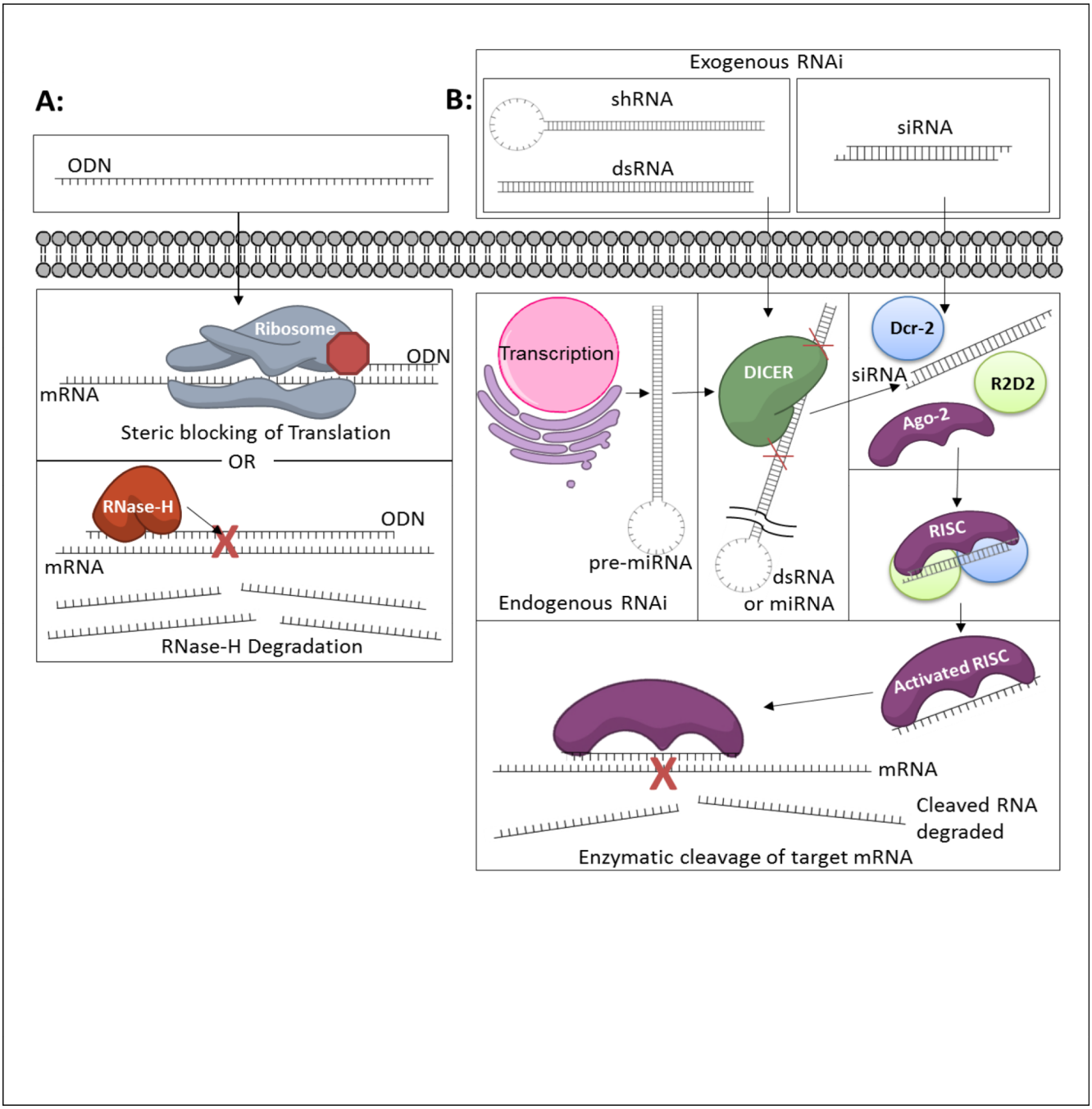

Figure 2. Mechanisms of RNA Interference

(A) ODN silencing is believed to be induced by sterically blocking translation through hybridization with mRNA and RNAse-H mediated degradation of both the ODN and mRNA (B) Proposed mechanisms for RNA interference by miRNA, shRNA, dsRNA, and siRNA. Endogenous miRNA, which is made transcriptionally, and exogenously delivered shRNA/dsRNA must all be first processed into siRNA, double stranded RNA molecules around 20 base pairs in size. Exogenous siRNA can also be delivered that circumvents the need for dicer processing. siRNA is loaded onto the RISC complex and mediates degradation of mRNA complementary to the antisense siRNA strand. For each type of therapeutic RNAi,

the exogenous RNA must reach the cytoplasm to interact with mRNA and other intracellular machinery required for gene silencing.

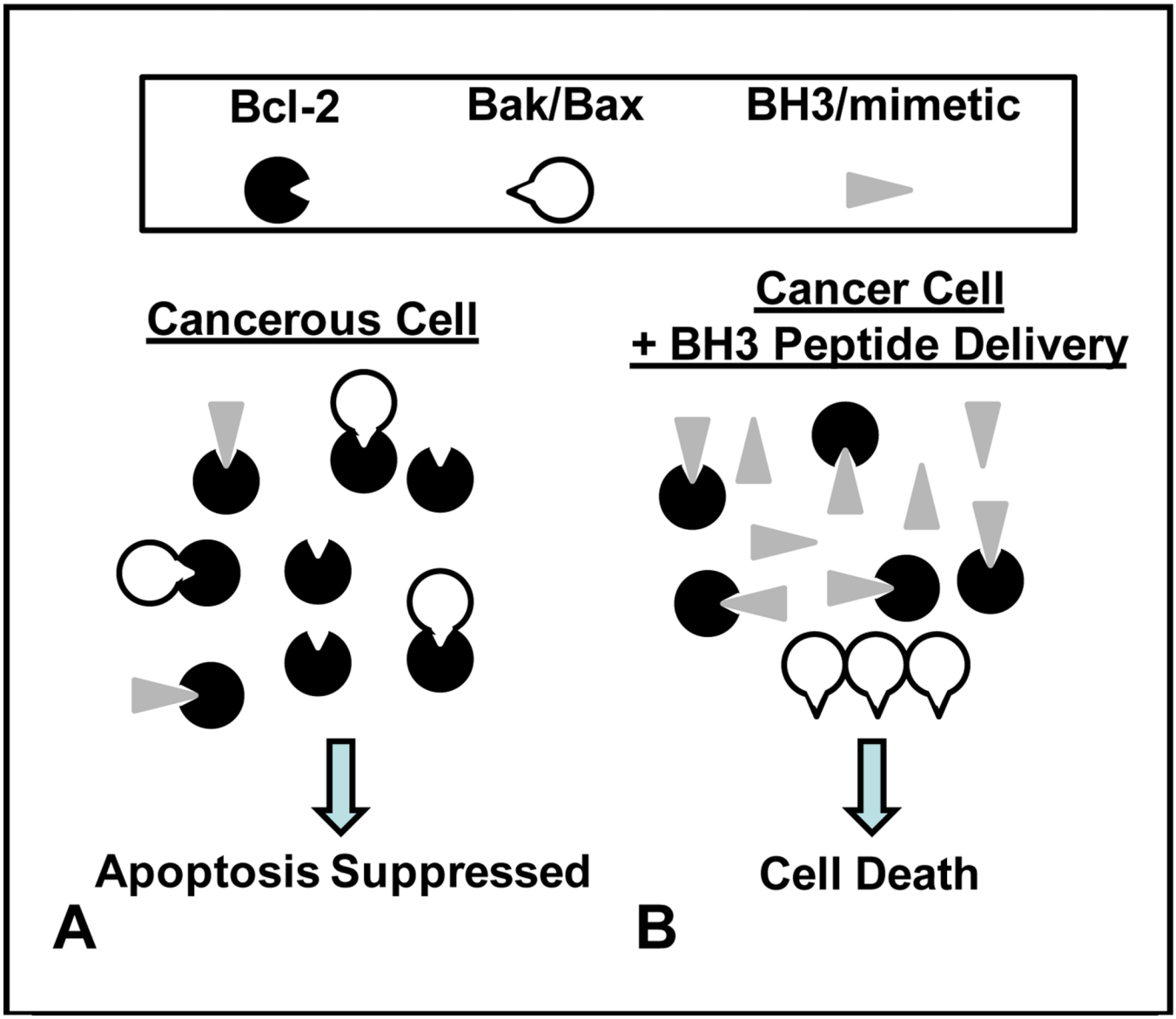

Figure 3. Model representation of BH3-only protein peptidomimetic cancer cell pro-apoptotic activity

Numerous cancers overexpress anti-apoptotic Bcl-2-like proteins, and BH3 domain-derived peptides can be utilized to antagonize Bcl-2-like protein activity to indirectly activate Bak/Bax and overcome apoptotic resistance.

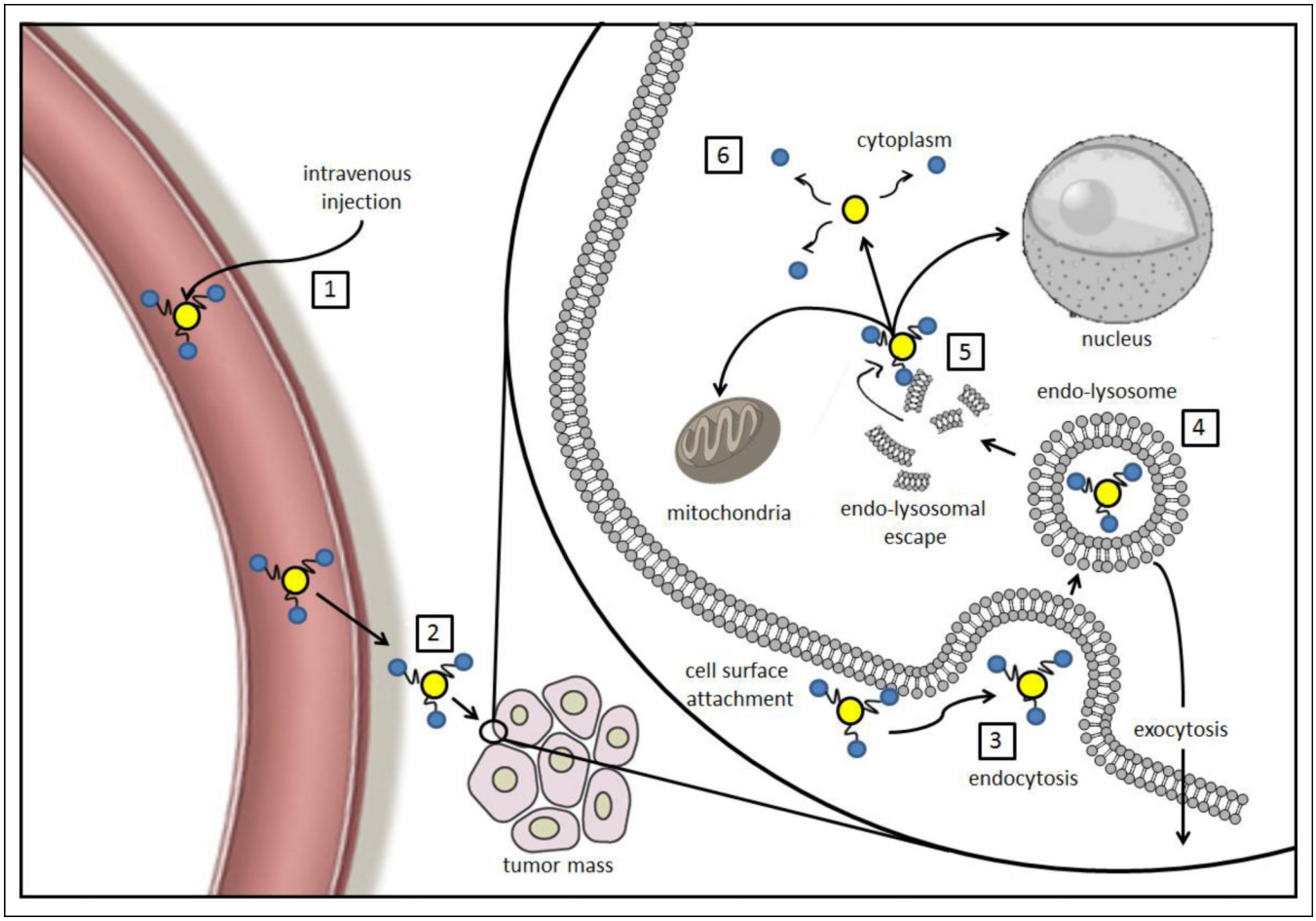

Figure 4. Intracellular delivery barriers

1) Upon entering systemic circulation, the drug formulation must protect the biomacromolecule from enzymatic degradation and serum protein destabilization and/or opsonization. 2) Drug circulation half-life needs to be long enough such that the drug persists in the vasculature until it accumulates (through specific targeting or nonspecifically thorough the enhanced permeation and retention effect) in the tumor tissue. 3) The drug must interact with the cell membrane to initiate internalization, which can result in uptake into an endosomal (or pinocytic) vesicle. 4) If no endosomal escape mechanism is present, the drug can be degraded in the lysosome, remain in the late endosome, or undergo trafficking for exocytosis. 5) If the drug is able to escape the endo-lysosomal pathway, it is released where it can diffuse to molecular targets in the cytoplasm. 6) If attached to a carrier or loaded within a nanoparticle such as a liposome, polyplex, etc., the drug may need to be released from this formulation to become bioavailable.

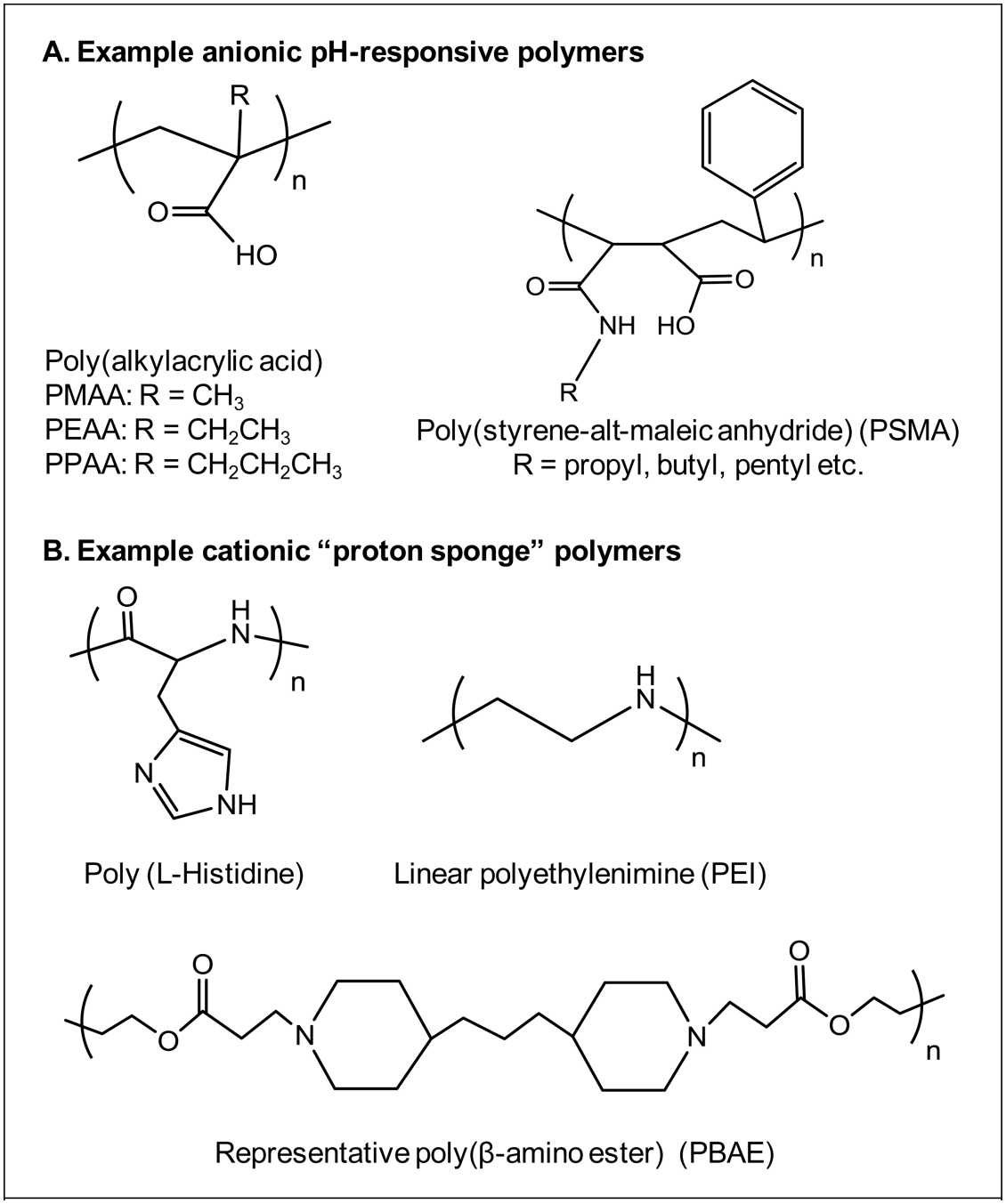

Figure 5. Chemical structures of example pH-responsive "smart" polymers used for intracellular drug delivery

(a) Anionic polymers thought to become more hydrophobic and lipophilic in acidic environments. (b) Cationic amine-containing polymers believed to disrupt endo-lysosomes through the proton sponge mechanism.

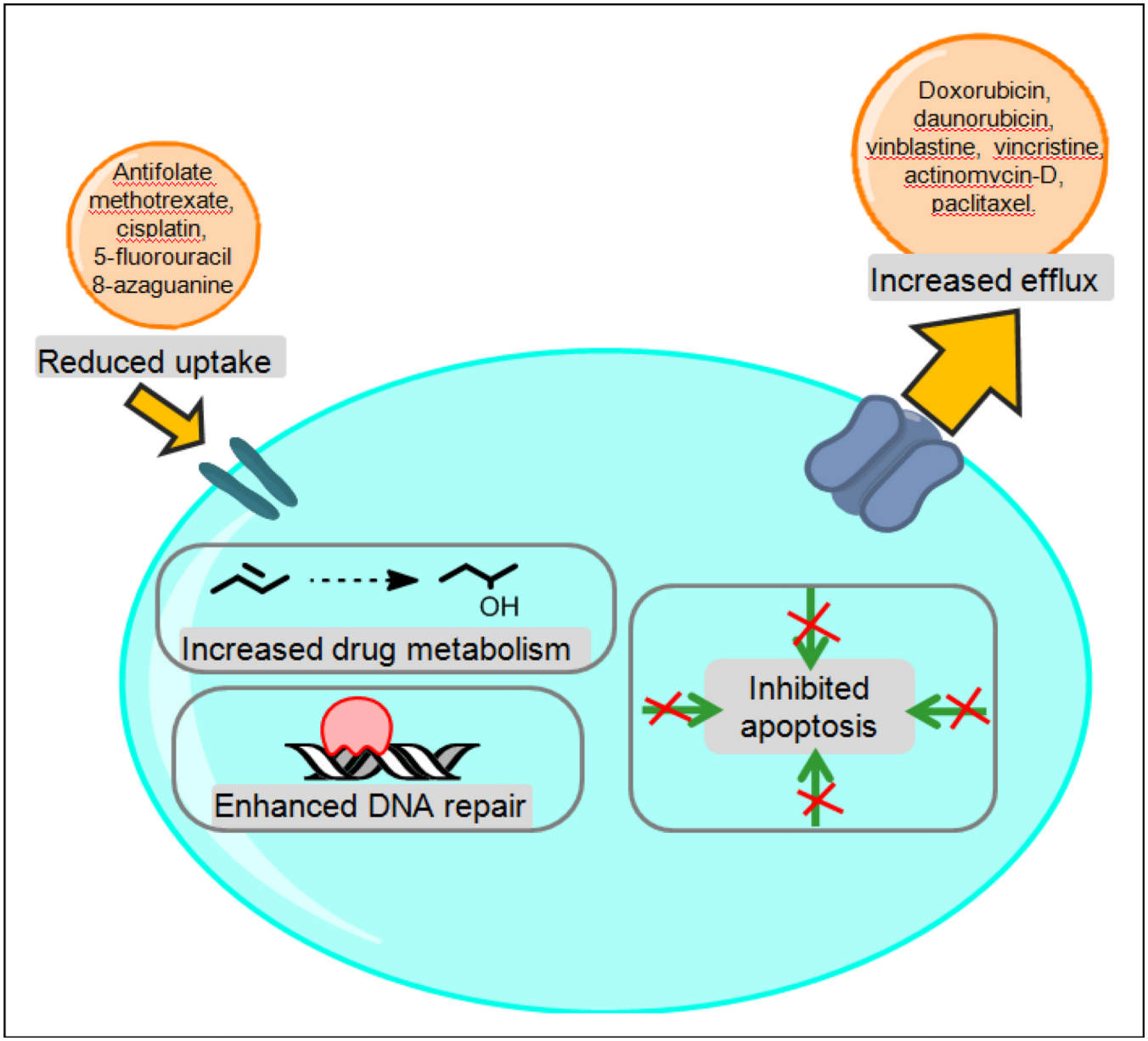

Figure 6. Multidrug Resistance

Examples of cellular characteristics that enable cancer cell multidrug resistance to chemotherapeutics include reduced uptake, increased metabolism, and increased efflux of the drugs. Chemotherapeutics affected are also summarized.

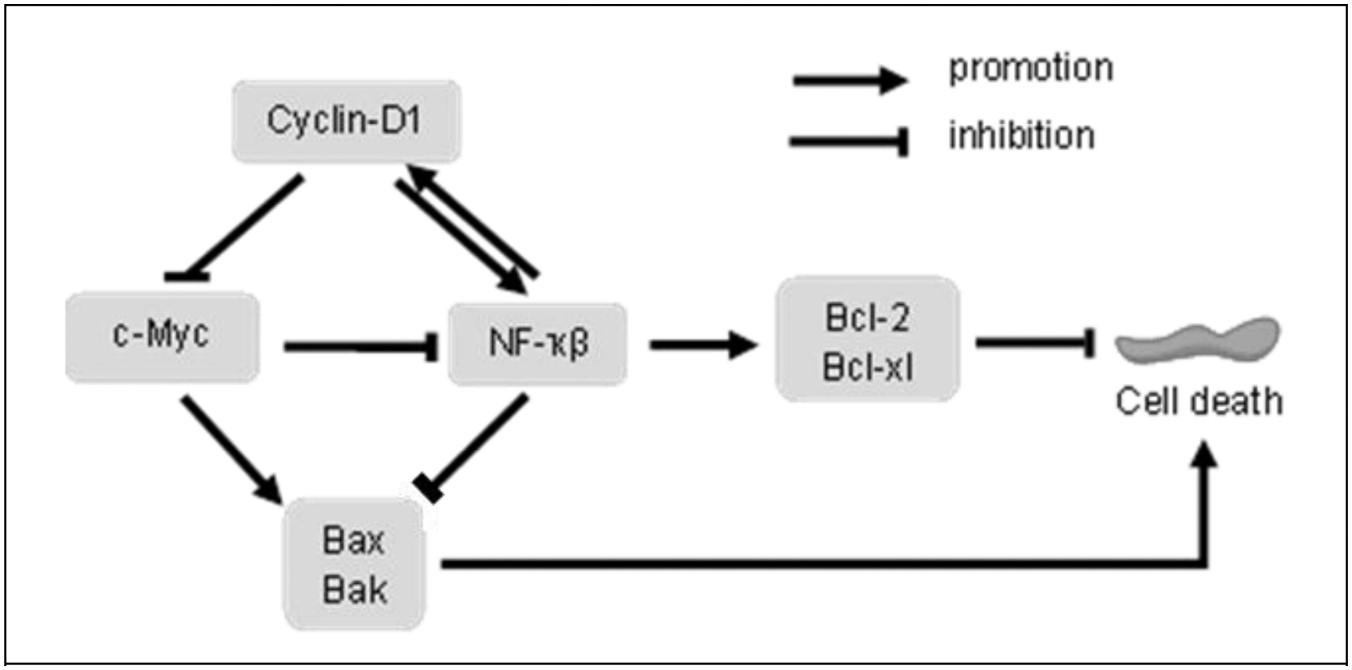

Figure 7. Relationships of some of the genes involved in MDR phenotype

Table 1

Cell Penetrating Peptides

Peptide (abbreviation)	Sequence	Sequence Origin	Ref
Penetratin (PEN, pAntp)	RQIKIWFQNRRMKWKK	*Drosophila* antennapedia homeodomain	[118]
Trans-activating transcriptional factor (TAT)	GRKKRRQRRRPPQ	Human Immunodeficiency Virus type I (HIV-1)	[118]
pVEC	LLIILRRRIRKQAHAHSK	Murine vascular endothelial cadherin	[118]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Galanin (neuropeptide) and mastoporan (toxic peptide in wasp venom)	[118]
YARA	YARAAARQARA	Optimized TAT transduction domain	[320]
WLR	WLRRIKAWLRRIKAWLRRIK	synthetic	[320]
VP22	MTSRRSVKSGPREVPRDEYEDLYYIPSSGMAS PDSPPDTSRRGALQTRSRQRGEVRFVQYDESD YALYGGSSSEDDEHPEVPRTRRPVSGAVLSGP GPARAPPPPAGSGGAGRTPTTAPRAPRTQRVA TKAPAAPAAETTRGRKSAQPESAALPDAPAST APTRSKTPAQGLARKLHFSTAPPNPDAPWTPR VAGFNKRVFCAAVGRLAAMHARMAAVQLW DMSRPRTDEDLNELLGITTIRVTVCEGKNLLQ RANELVNPDVVQDDAATATRGRSAASRPTER PRAPARSASRPRRPVE	Herpes Simplex Virus type I (HSV-1)	[321]
SynB vector	RGGRLYRRRFVVGR	Protegerin-I analogue without cysteine residues	[322]
Diatos Peptide Vector 3 (DPV3)	RKKRRRESRKKRRRES	Superoxide dismutase	[117]
Diatos Peptide Vector 6 (DPV6)	GRPRESGKKRKRKRLKP	Platelet-derived growth factor (PDGF)	[117]
Diatos Peptide Vector 7 (DPV7)	GKRKKKGKLGKKRDP	Epidermal-like growth factor	[117]
Diatos Peptide Vector 7b (DPV7b)	GKRKKKGKLGKKRPRSR	Epidermal-like growth factor	[117]
Diatos Peptide Vector 3/10 (DPV3/10)	RKKRRRESRRARRSPRHL	Superoxide dismutase and intestinal mucin	[117]
Diatos Peptide Vector 10/6 (DPV10/6)	SRRARRSPRESGKKRKRKR	Intestinal mucin and PDGF	[117]
Diatos Peptide Vector 1047 (DPV1047)	VKRGLKLRHVRPRVTRMDV	Apolipoprotein B and anti-DNA antibody	[117]
Diatos Peptide Vector 10 (DPV10)	SRRARRSPRHLGSG	Intestinal mucin	[117]
Diatos Peptide Vector 15 (DPV15)	LRRERQSRLRRERQSR	Cationic antimicrobial protein 37 (CAP37)	[117]
Diatos Peptide Vector 15b(DPV15b)	GAYDLRRRERQSRLRRRERQSR	Cationic antimicrobial protein 37 (CAP37)	[117]
R8	RRRRRRRR	Oligoarginine (synthetic)	[114]
R12	RRRRRRRRRRRR	Oligoarginine (synthetic)	[120]
IRQ	IRQRRRR	Oligoarginine derivative (synthetic)	[323]
basic fibroblast growth factor 1 (HbFGF-1)	NYKKPKL	Nuclear localization sequence in HbFGF-1	[324]
basic fibroblast growth factor 2 (HbFGF-2)	HFKDPKR	Nuclear localization sequence in HbFGF-2	[324]
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	HIV Gp41-SV40 nuclear localization sequence	[121]
Sweet arrow peptide (SAP)	VRLPPPVRLPPPVRLPPP	Proline rich motif	[121]

Peptide (abbreviation)	Sequence	Sequence Origin	Ref
hCT (9–32)	LGTYTQDFNKTFPQTAIGVGAP	Human calcitonin	[121]
Pep-1	KETWWETWWTEWSQPKKKRKV	Trp-rich motif-SV40 nuclear localization sequence	[121]

Table 2

Fusogenic Peptides

Peptide (abbreviation)	Sequence	Sequence Origin	Ref
Model amphipathic peptide (MAP)	KALAKALAKALA	Chimeric	[121]
GALA	WEAALAEALAEALAEHLAEALAEALEALAA	Model amphipathic peptide derivative	[135]
KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	Model amphipathic peptide derivative	[141]
EALA	EALAEALAEALA	Chimeric	[142]
4(6)	Ac-LARLLARLLARLLRALLRALLRAL	Synthetic amphipathic peptide	[143]
Hel 11-7	KLLKLLLKLWKKLLKLLK	Synthetic amphipathic peptide	[143]
INF7	GLFEAIEGFIENGWEGMIWDYG	23 mer Hemagglutinin (HA-2) derivative	[137]
JTS-1	GLFEALLELLESLWELLLEA	INF7 mimic	[137]
ppTG1	GLFKALLKLLKSLWKLLLKA	JTS-1 mimic	[144]
ppTG20	GLFRALLRLLRSLWRLLLRA	JTS-1 mimic	[144]
LAH4 Analogues	KKALLALALHHLAHLALHLALALKK	Histidine rich amphipathic peptide	[145]
β-amyloid (29–42)	GAIIGLMVGGVVIA	Transmembrane β-amyloid protein derivative	[146]
Influenza HA-2	GLFGAIAGFIENGWEGMIDGWYG	N-terminal sequence of influenza hemagglutinin subunit HA-2	[140]
Influenza derivative I	GLFQAIAGFIQNGYQGMIDGGGC	Hemagglutinin (HA-2) mimic	[138]
Influenza derivative II	GLFEAIAEFIEGGWEGLIEGCA	Hemagglutinin (HA-2) mimic	[139]
Mellitin	GIGAVLKVLTTGLPALISWIKRKRQQ	Peptide component of bee venom	[148]
Penetratin (PEN, pAntp)	RQIKIWFQNRRMKWKK	*Drosophila* antennapedia homeodomain	[118]
Trans-activating transcriptional factor (TAT)	GRKKRRQRRRPPQ	Human Immunodeficiency Virus type I (HIV-1)	[118]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Galanin (neuropeptide) and mastoporan (toxic peptide in wasp venom)	[118]

Table 3

Application of Cationic Polymeric Carriers to Facilitate Endosomal/Lysosomal Escape.

Cationic Polymer	Gene	Cell	Efficacy	Ref
Cationic polymeric nanoparticles				
PEI	STAT3	B16 melanoma	~5% gene silencing (25 nM);~25% gene silencing and 13% cell death (50 nM).	[325]
PEI-StA	STAT3	B16 melanoma	~53% gene silencing (25 nM);~68% gene silencing and 40% cell death (50 nM).	[325]
PDMAEMA	VEGF	PC3 prostate cancer cell	68 % gene silencing (N/P = 2) 97 % gene silencing (N/P = 4)	[173]
PDMAEMA-*b*-PDbB complex with PSMA	Plk1	MDR Ovarian cancer cell	>70% transfection, 50% gene silencing, and 5%–10% apoptosis	[272]
PEI-*b*-PEG-RGD	Luciferase gene VEGF-R2	Neuroblastoma In vivo, mice	90% gene silencing Tumor size barely increased (6 fold bigger for untreated and ~12 fold bigger for siRNA negative control	[326]
EHCO	Luciferase	CHO-d1EGFP	84% cellular uptake and 87% gene silencing	[211]
Biodegradable cationic polymeric nanoparticles				
PAMAM dendrimer complexed with PEG-*b*-P(PrMA-co-MAA	Bcl-2	PC-3 prostate cancer cell	62% gene silencing (50 nM)	[226]
PAMAM dendrimer	Hsp27	PC-3 prostate cancer cell	75% gene silencing (50 nM) and ~45% cell apoptosis	[327]
PEO-*b*-PBAE	MDR-1	SKOV3 human ovarian adenocarcinoma	Effective gene silencing (100 nM) and 10 – 25% greater cell apoptosis induced in SKOV3 cells, <5% greater cell apoptosis induced in SKOV3 MDR cells	[328]
PBAE	Luciferase gene	Hepatoma, primary hepatocytes	~38%–75% gene inhibition and 10%–20% cell apoptosis in hepatoma 50%–70% gene inhibition in primary hepatocytes	[204]
PHPMA-MPPM TMC	Luciferase gene	H1299 lung cancer cells	30%–40% gene silencing (compared with 50% with PDMAEMA nanoparticle and 60% with lipofectamine)	[329]

EHCO: N-(1-aminoethyl)iminobis[N-(oleicyl-cysteinyl-histinyl-1-aminoethyl)propionamide; RGD: Arg-Gly-Asp; Hsp27: heat-shock protein 27; PHPMA-MPPM: poly((2-hydroxypropyl)methacrylamide 1-methyl-2-piperidine methanol); TMC: *O*-methyl-free *N,N,N*-trimethylated chitosan; PBME: Poly(β-amino esters); PDbB: butyl methacrylate; PSMA: poly(styrene-alt-maleic anhydride). Concentrations provided in the "Efficacy" column refer to siRNA.

Table 4

In vitro Success at Inducing Apoptosis through siRNA Knockdown.

Gene Target	Delivery Method	Cell line	Effect	Ref
Anti-Apoptotic				
Bcl-2	siRNA vector Cationic Lipid Lipofectamine™	HeLa HeLa, Lung adenocarcinoma, hepatoma, ovarian carcinoma, ocular melanoma Melanoma, renal carcinoma	Stopped growth Stopped growth 25–35 % apoptosis	[250] [251] [253]
Bcl-xL	X-tremeGENE kit™	PHK, HaCaT	Growth inhibition and apoptosis	[330]
BAXi	Oligofectamine®	Prostate carcinoma	45% apoptotic cells	[331]
Survivin	Oligofectamine® Plasmid	Human sarcoma ovarian carcinoma, ocular melanoma Melanoma, renal carcinoma	10% apoptosis 45–70% apoptosis	[258] [253]
Livin	siRNA vector pSUPER	HeLa HeLa	8% apoptotic cells 4% apoptotic cells	[257] [332]
XIAP	Lipofectamine™	Ovarian cancer	75% apoptosis	[260]
c-myc	Lipofectamine™	MCF-7	50% apoptosis	[267]
Oncogenes				
HER2/neu	Oligofectamine®	SKBr3, MCF7/HER2 tumor cell lines	20–50% apoptosis	[267]
Viral E6 oncogene	pSUPER	HPV+ cervical carcinoma	Massive apoptosis of susceptible cells	[333]
Lyn Kinase (oncoprotein)	electroporation	Chronic Myelogenous Leukemia	40% apoptosis	[334]
PLK1	Oligofectamine® Plasmid vector	Breast cancer, HeLa, colon cancer, lung cancer HeLa, prostate cancer, human glioblastoma, dermal fibroblasts	13–50% apoptosis <5% apoptosis	[271] [335]
Protein kinase B	Lipofectamine™	Bone marrow cells, HEK 293	20% apoptosis	[336]
HDAC3	Oligofectamine®	HeLa	15% apoptosis	[337]
B-RAF	Thymidine	A375 Colo829 WM-266-4	23–52% apoptosis	[338]
OPA1	Oligofectamine®	HeLa NIH-OVCAR-3	38% apoptosis	[339]
m-bcr/abl	siRNA vector	K562 leukemic cell	7.5 fold increase in apoptosis	[340]

Table 5

In vivo Success at Inducing Apoptosis through siRNA Knockdown.

Gene Target	Method	Cell line / Animal Model	Effect	Ref
Anti-Apoptotic				
c-myc	Anisamide-targeted nanoparticles Plasmid polymerase III promoter Protamine-antibody fusion protein	Melanoma in mice MCF-7 in mice B16 melanoma in mice	Tumor growth decreased by 75% relative to control Increased tumor latency Tumor growth decreased by 75% relative to control	[262, 265, 266] [263] [264]
Bcl2	*In vivo*-jetPEI™	Melanoma in mice	15–50% apoptosis, decrease in tumor growth	[252]
BAXi	Superfect	HT1080 and B16F10 in mice	Reduces cancer metastasis	[341]
Survivin	DharmaFect®	A549/DDP in mice	Tumor growth decreased by 40% relative to control	[259]
XIAP	Lipofectamine™	MCF-7 cells in mice	Tumor growth decreased by 75% relative to control	[261]
Oncogenes				
Coagulation tissue factor mTF	Lipofectamine™ Ex vivo transfection	B-16 cells in Female C57BL/6 mice	Tumor growth did not vary significantly	[342]
PAR-1	Neutral liposomes (DOPC)	A375SM human melanoma cell line Female athymic nude mice (NCr-nu)	Tumor growth decreased by 75% relative to control, reduced invasion of cancer cells	[343]
HIF-1alpha	shRNA – Lipofectamine™ shRNA-Tf-PEI	A375 and A875 cells Nude mice	A375 – 50% reduction in tumor size A875 – no effect	[344]
STAT3	Lipid-substituted PEI	Murine B16.F10 cells Male C57Bl/6 mice	Significant increase in caspase 3 activity, tumor growth decreased by 70% relative to control	[325]
S100A4	pS100A4-shRNA	gastric cancer cell line, BGC823, nude mice	Tumor growth decreased by 75% relative to control	[345]
WT1	Aerosol PEI	B16 F10 mouse melanoma	Tumor growth decreased by 80%, survival time increased	[346]
Plk1	*In vivo* jetPEI™	C666-1 cells in mice	Increase to 50% multinuclear cells and decrease in tumor growth	[270]