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Polymer-Drug Conjugates as Modulators of Cellular Apoptosis

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ABSTRACT

The successful clinical application of polymer-protein conjugates (PEGylated enzymes and cytokines) and the promising results arising from clinical trials with polymer-bound chemotherapy (eg, doxorubicin or paclitaxel) have established their potential to reduce toxicity and improve activity in chemotherapy-refractory patients. Furthermore, and more important, they have also provided a firm foundation for more sophisticated second-generation constructs that deliver the newly emerging target-directed bioactive agents (eg, modulators of apoptosis, cell cycle, anti-angiogenic drugs) in addition to polymer-based drug combinations (eg, endocrine therapy and chemotherapy). This review will focus on polymer-drug conjugate modulators of cellular apoptosis to be used as single pro-apoptotic (eg, cancer) or anti-apoptotic (eg, ischemia) agents or as a combination therapy.

KEYWORDS: Polymer-drug conjugates, apoptosis modulators, targeted delivery

INTRODUCTION

Polymer-drug conjugates are nano-sized hybrid constructs that covalently combine a bioactive agent with a polymer to ensure not only its efficient delivery to the required intracellular compartment but also its availability within a specific period of time.[1] It has already been demonstrated that polymer-drug conjugation promotes tumor targeting by the enhanced permeability and retention effect[2] and, at the cellular level following endocytic capture, allows lysosomotrophic drug delivery. Consequently, polymer-drug conjugates have the potential to improve the therapy of common drug-resistant solid tumors by reducing toxicity and improving activity in chemotherapy-refractory patients. These multicomponent constructs have been already transferred to clinics as anticancer agents, either as single agents or as elements of combinations. A vast amount of literature has been accumulating over the last 30 years, and exhaustive reviews have been written on polymer conjugates.[3-6] The promising results arising from clinical trials with polymer-bound chemotherapy have laid a firm foundation for more sophisticated second-generation constructs delivering newly emerging target-directed treatments (eg, cell cycle or apoptosis modulators)[7,8] and polymer-drug combinations.[9] The use of polymer-drug conjugates in combination therapy is seen as an important opportunity to enhance disease response rates.

Apoptosis is an interesting biological process because it is implicated in a wide variety of biological systems, including normal cell turnover, the immune system, and the immune system's association with different diseases. Inappropriate apoptosis is involved in human pathologies, including neurodegenerative diseases such as Alzheimer's and Huntington's, ischemia, autoimmune disorders, and several forms of cancer (Table 1).[10] Therefore, there is increased interest in defining new pharmacological targets that could control apoptosis pathways, which in turn would offer new opportunities for the discovery and development of drugs.[11-13]

Diverse apoptotic stimuli, including activation of cell surface death receptors, anticancer agents, irradiation, lack of survival factors, and ischemia (reviewed in Strasser et al[14]), induce signaling cascades that all activate a family of cysteine aspartyl proteases called caspases. These proteases execute the apoptotic process. Effector caspases (eg, caspases-3 and -7) are responsible for the disassembly of cellular components, while initiator caspases (eg, caspases-8, -9, and -10) are responsible for the activation of the effector caspases. While different apoptotic stimuli activate different initiators, these initiators activate a common set of effector caspases. Some apoptotic signals activate the mitochondria-mediated or intrinsic pathway that uses caspase-9 as its initiator. Caspase-9 activation is triggered by the release of pro-apoptotic proteins from the mitochondrial intermembrane space to the cytosol, in particular cytochrome c and Smac/DIABLO.[15,16] The formation of the macromolecular protein complex named apoptosome is a key event in this pathway. The apoptosome is a holoenzyme multiprotein complex formed by cytochrome c-activated apoptotic protease-activating factor (Apaf-1) and procaspase-9. In this macromolecular complex, apoptosome-associated caspase-9 is activated and then, in turn, activates effector caspases.[17-19]

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Table 1. Human Diseases Involving a Defective Regulation of Apoptosis, Programmed Cell Death Mechanism[7,10]

Deregulation	Disease
Insufficient apoptosis	• Cancer
	• Persistent infections
	• Restenosis
	• Autoimmunity
Excessive apoptosis	• Cardiovascular diseases: myocardial infarction, stroke, heart failure
	• Neurodegenerative diseases: stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, amyotrophic lateral sclerosis, traumatic brain injury and/or spinal cord injury
	• Inflammation, sepsis
	• Human immunodeficiency virus (HIV)
	• Type I diabetes
	• Organ transplants
	• Alopecia

Apoptosis-modulating treatments could be classified as pro-apoptotic (more commonly known as anticancer therapies) and anti-apoptotic. The anti-apoptotic opportunities could be further subdivided into acute and chronic types of disease. However, it is important to note that anti-apoptotic drugs for chronic diseases are unlikely to be available for many years owing to the tolerance of patients to drugs that affect normal physiological apoptotic pathways.

Although a short historical overview will be given, this review focuses on polymer-drug conjugate modulators of apoptosis, a key cellular mechanism, used as single agents or as a combination therapy.

Pro-Apoptotic Polymer Drug Conjugates: Cancer as a Target

Polymer-Drug Conjugates Carrying Classical Anticancer Agents

Over the past decade 11 polymer-drug conjugates have entered clinical trials as intravenously administered anticancer agents (extensively reviewed in Duncan,[3] Satchi-Fainaro et al,[4] and Vicent and Duncan[5]). Most of these conjugates are known to exert their antitumor activity by inducing apoptosis through the traditional anticancer drug attached. However, certain chemotherapeutic agents, such as doxorubicin (Dox), paclitaxel (PTX), or camptothecin (CPT), work by initially acting nonspecifically by either damaging DNA (Dox and CPT) or disrupting the cytoskeleton (PTX).[20]

The N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-Dox conjugate PK1 (also named FCE28068) was the first synthetic polymer conjugate to enter phase I in 1994.[21] The molecular mechanism of action of PK1 in comparison to free Dox has been studied thoroughly by different research groups, but a consensus has not yet been reached. The kinetic complexities of in vitro experiments involving these macromolecules make design and interpretation of such studies particularly challenging. Conjugates and free drugs have such different cellular pharmacokinetics and, moreover, all conjugates contain 1.0 to 0.01% free drug. Consequently, some studies have suggested that PK1 acts by a strong activation of apoptosis signaling pathways,[22] while others have suggested that the primary mechanism of cell death induced by PK1 is necrosis.[23] On the other hand, there is growing evidence that the early antitumor activity in vivo occurs via cytotoxic or cytostatic drug action but that the secondary immunostimulatory action of circulating low levels of conjugate augments this effect.[24,25]

Since PK1, 5 more HPMA copolymer conjugates containing established chemotherapy, such as PTX, platinates, or CPT, and 2 HPMA copolymer-derived gamma camera imaging agents have also progressed to clinical testing.[26] Conjugates based on other polymeric carriers, such as poly(ethylene glycol) (PEG), poly(glutamic acid) (PGA), or polysaccharides, are also now found in clinics.[3] Currently, polyglutamate-PTX (CT-2103 or Xyotax) is the most clinically advanced polymer-anticancer conjugate (Figure 1). This PGA conjugate was first designed by Li et al[27] and further developed by Cell Therapeutics Inc (www.cticseattle.com) up to the phase III clinical trials either as a single

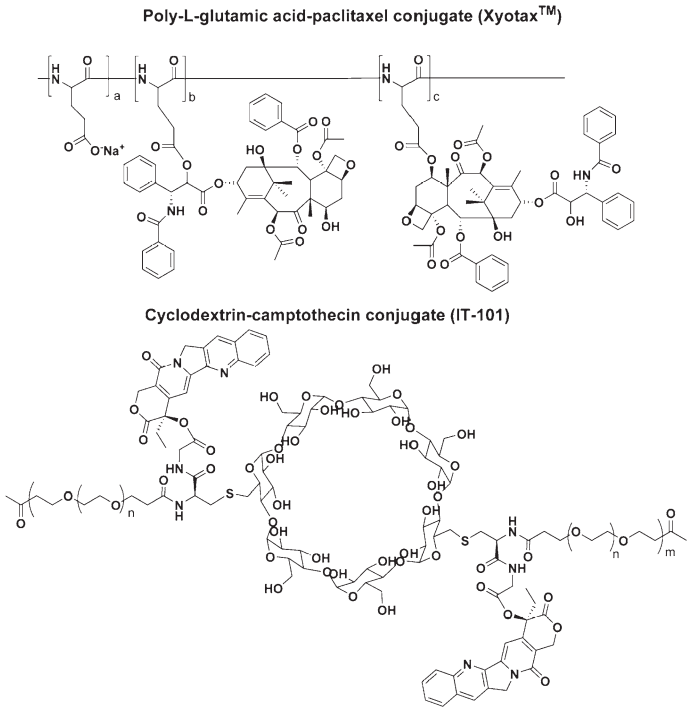

Figure 1. Examples of polymer-drug conjugates carrying classical anticancer agents with pro-apoptotic activity.

agent or in combination with standard chemotherapy or radiotherapy as the treatment for several types of cancer (non-small cell lung cancer [NSCLC] and ovarian, esophageal, and gastric cancer).[28] Xyotax is expected to be the first polymer-anticancer conjugate to enter the market for the treatment of NSCLC in women.[29,30] In anticipation of regulatory approval, Cell Therapeutics Inc announced on September 18, 2006, an exclusive worldwide licensing agreement with Novartis (www.cticseattle.com or www.novartis.com) for the development and commercialization of Xyotax. In this conjugate, PTX is linked to the carrier via an ester bond. This type of linkage had proved unsuccessful for HPMA copolymer CPT and HPMA PTX, since it led to premature drug release by blood esterases. However, the presence of a different polymeric carrier (PGA as opposed to HPMA copolymer), as well as the high drug loading (~37% wt/wt), resulted in a stabilization of the linker, probably because of the conformation adopted in solution. Indeed, it was shown that the main drug release occurred subsequent to polymer degradation by the lysosomal enzyme cathepsin B. Morphological analysis and biochemical characterization have demonstrated that both PGA-PTX conjugate and free PTX possess similar abilities to induce apoptosis and that p53 did not appear to play a significant role in drug-induced cell death with either compound. It has also been demonstrated that both agents induced a characteristic G(2)/M arrest in the cell cycle, consistent with the disturbance of microtubule polymerization as their mechanism of action.[31]

Another PGA conjugate, a PGA-CPT conjugate (CT-2106),[32] has also entered phase I/II trials in patients with advanced malignancies. Stable disease was seen in 6 of the 24 treated patients, and the conjugate was well tolerated. CPT is a classical anticancer agent that induces cell death by converting DNA topoisomerase I into a DNA-damaging agent; formation of covalent and nonreversible topoisomerase I-DNA complexes during DNA replication results in strand breaks and subsequent induction of apoptosis.[33] The conjugation of CPT to a polymeric carrier through an appropriate linker clearly enhances its anticancer efficacy, as demonstrated by PGA-CPT (CT-2106), PEG-CPT (pegamotecan, EZ-246),[34] and cyclodextrin-CPT (IT-101) conjugates (Figure 1).[35]

IT-101 is a conjugate of camptothecin and a linear cyclodextrin-based polymer (CDP). The components of CDP are β-cyclodextrin and PEG. Pharmacokinetic and preclinical studies have demonstrated that this conjugate exhibits a longer plasma half-life and better distribution to the tumor tissue than does CPT alone. Furthermore, IT-101 shows good tolerability and potent antitumor activity against a wide range of solid tumors.[35-37] A phase I safety and pharmacokinetic study of IT-101 in the treatment of advanced solid tumors sponsored by Insert Therapeutics (www.insertt.com) is currently recruiting patients (www.clinicaltrials.gva.gov). This will be an open-label dose-escalation study of IT-101 administered in patients with solid tumor malignancies. Patients who meet the inclusion/exclusion criteria will receive a weekly injection of IT-101 followed by a 1-week rest period.

Although Enzon Pharmaceuticals Inc (www.enzon.com) announced the discontinuation of Prothecan/pegamotecan in February 2005 (because of strategic analysis after a phase IIb trial in patients with gastric or gastroesophageal cancers), Minko et al clearly demonstrated that conjugation of CPT to PEG markedly increased its pro-apoptotic activity. This apoptosis enhancement was assessed by TUNEL labeling, caspase activity, and the expression of genes encoding Bcl-2, Apaf-1, and the caspase-3 and caspase-9 proteins.[38-40] To further enhance the observed apoptosis induction, a biotin moiety (uptake via the sodium-dependent multivitamin transporter) was covalently introduced, leading to the creation of a CPT-PEG-biotin conjugate.[38] Biotinylation of the PEG led to an increase in CPT toxicity of more than 60 times in sensitive cells and almost 30 times in resistant cells when compared with an equivalent concentration of free CPT or CPT-PEG. Enhanced overexpression of genes encoding Apaf-1, caspase-3, and caspase-9, together with down-regulation of the Bcl-2 gene, was observed for the CPT-PEG-biotin conjugate.

Polymer-Drug Conjugates Carrying Novel Target-Directed Anticancer Therapy

Although polymer anticancer conjugates are showing promise in clinical trials[3] and the first conjugate is expected to appear on the market in 2007,[28] recent clinical data have underlined the complexity of the pharmacokinetics of these macromolecular drugs. We still need to better understand the pharmacokinetic-pharmacodynamic relationships of conjugates in vivo.[41] The second-generation conjugates currently emerging are using new polymer platforms, polymer-bound combination therapy, and new molecular targets in an attempt to further enhance activity and circumvent resistance. The increasing understanding of cancer's molecular basis has led to the discovery of newly emerging target-directed anticancer agents such as tumor-selective apoptosis-inducing agents, modulators of the cell cycle, signal transduction inhibitors, and anti-angiogenic drugs.[42-45]

The first polymeric anti-angiogenic conjugate, HPMA copolymer-fumagillol (TNP-470), caplostatin has shown considerable promise in preclinical studies (Figure 2).[46] TNP-470 is known to produce apoptosis in vascular endothelial cells, which has been proved by TUNEL experiments.[47-49] Drug conjugation prevents TNP-470 from crossing the blood-brain barrier, thus preventing its inherent

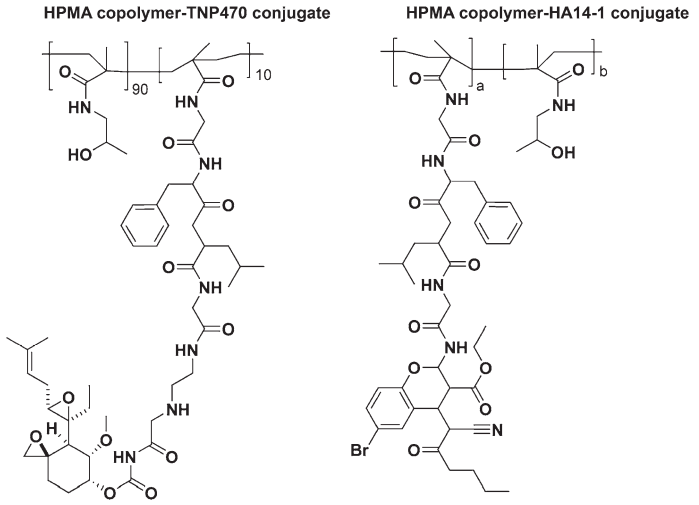

Figure 2. Examples of polymer-drug conjugates carrying novel target-directed anticancer therapy with pro-apoptotic activity. HPMA indicates N-(2-hydroxypropyl)methacrylamide.

neurotoxicity. A recent study showed the eradication of human colon carcinoma in mice when caplostatin was combined with the monoclonal antibody bevacizumab (Avastin). Following the same idea of targeting tumor neovasculature, Mitra et al[50] recently described a novel polymer-peptide conjugate, HPMA copolymer-RGD4C-Tc-99m conjugate, capable of targeting tumor angiogenic vessels and delivering adequate radiotherapy to arrest tumor growth. In a xenograft model of human prostate carcinoma, this targeted conjugate showed significant tumor accumulation. Additionally, a histopathological examination revealed increased apoptosis in the treated tumors with no acute signs of radiation-induced toxicity to other organs. Other similar examples are the PEGylated cyclic arginine-glycine-aspartic acid (RGD) radiotracers (64-Cu-DOTAPEG-RGD and ^{125}I-RGD-mPEG).[51,52]

Focusing on tumor-selective apoptosis-inducing agents, it is well known that the Bcl-2 protein plays a key role in the mitochondrial-dependent apoptosis pathway and is therefore considered an interesting therapeutic target in tumor pathogenesis.[53] Several low-molecular-weight Bcl-2 inhibitors have already been identified, but their efficacy in vivo has been very poor, mainly because of solubility and cell membrane permeability problems. Conjugation to a hydrophilic polymeric carrier could overcome these drawbacks.[54] Recently, the first bioconjugate of this type has been developed, HPMA copolymer-HA14-1 conjugate (Figure 2).[55] The conjugate's in vivo studies demonstrated a much greater efficacy than free drug's. After intraperitoneal administration, HA14-1 conjugates were capable of suppressing tumor growth by 50%. Whereas activated caspase-9 protein was detected in tumors treated with the bioconjugate, none was found in either normal organs or tumors treated with a control polymer.

Reactive oxygen species (ROS) are potentially harmful byproducts of normal cellular metabolism that directly affect cellular functions and survival.[56] It has also been reported that ROS induce apoptosis of many tumor cells in vitro via the activation of the caspase cascade. Therefore, targeting tumor cells by inducing oxidative stress, or targeting the cells that sensitize the tumor to oxidative insults, has been considered another interesting approach in cancer therapy. The synthesis of the PEG–zinc protoporphyrin (ZnPP) conjugate, a specific heme oxygenase (HO) inhibitor,[57-60] was developed with this objective in mind. PEG-ZnPP is found to induce cytotoxic effects by itself, as it makes cells more vulnerable to toxic insults. This conjugate is also able to greatly strengthen the toxicity induced by peroxides and anticancer agents, both in vitro and in vivo.[59] In some studies, PEG-ZnPP treatment produced a tumor-selective suppression of HO activity as well as an induction of apoptosis, possibly by increasing oxidative stress.[57-61]

Pro-Apoptotic Polymer-Drug Combination Therapy

The use of polymer-drug conjugates in combination therapy is seen as an important opportunity to enhance tumor response rates.[62] The polymeric carrier provides an ideal platform for the delivery of a cocktail of drugs simultaneously. We have recently reported the first endocrine-chemotherapy combination in the form of the model compound HPMA copolymer-aminoglutethimide-Dox (Figure 3).[9] It was discovered that the conjugate containing both drugs exhibited markedly enhanced cytotoxicity compared with HPMA copolymer-Dox, a conjugate that has already shown clinical activity in breast cancer patients,[21] whereas mixtures of polymer conjugates containing only aminoglutethimide (AGM), or only Dox, did not show a synergistic benefit. Complex cellular mechanisms appear to be responsible for

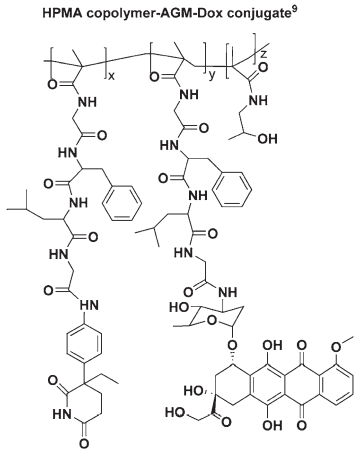

Figure 3. Structure of HPMA copolymer-AGM-Dox conjugate as example of pro-apoptotic polymer-drug combination therapy. HPMA indicates N-(2-hydroxypropyl)methacrylamide; AGM, aminoglutethimide; Dox, doxorubicin.

the increased antitumor activity of HPMA copolymer-AGM-Dox in vitro. Differences in the drug release profile were evident, and this conjugate caused a significant change in the expression of the anti-apoptotic protein Bcl-2. The presence of HPMA copolymer-Dox had no effect on Bcl-2 in MCF-7 and MCF-7ca cells, whereas decreased Bcl-2 was seen following incubation with the combination conjugate, suggesting that combining AGM and Dox leads to a synergistic effect that induces apoptosis; hence, the combination polymer shows increased activity. Further studies are needed to continue investigating these effects, but these observations underline the possibility of designing polymer-drug combinations for the improved treatment of breast and prostate cancer in the future.[63]

Another group prepared a branched PEG conjugate containing the combination of epirubicin (EPI) and nitric oxide (NO) (EPI-PEG-amino adipic acid (AD)-NO).[64] It is known that NO increases the antitumoral activity of several chemotherapies, while it provides protection against apoptosis induced by oxidative stress in both endothelial cells and cardiomyocytes. NO modulates the apoptotic properties (pro- and anti-) of chemotherapy agents. Therefore, the rationale for this combination is 2-fold. First, epirubicin and NO have a synergistic effect. In addition, NO displays a cardioprotective action, possibly counterbalancing the EPI-induced cardiotoxicity. In vitro studies showed that this conjugate induced apoptosis in Caco-2 cells at a higher level than did free EPI. In addition, the presence of NO on the conjugate conferred protection against EPI-mediated cardiotoxicity in adult cardiomyocytes. Researchers found that concentrations of PEG-EPI-NO that cause apoptosis in ~60% of colon cancer cells were not cytotoxic for the embryonic heart-derived cell line H9c2, adult cardiomyocytes, and human umbilical vein endothelial cells.[64]

Combining different polymer conjugates, each carrying a single therapeutic agent, has also been suggested. Using this concept of combination while also using targeting residues, Minko et al[40] investigated the feasibility of a 2-tier targeting of CPT-PEG conjugates to luteinizing-hormone releasing hormone (LHRH) receptors and cellular anti-apoptotic defense using LHRH and Bcl-2 homology 3 domain (BH3) peptides, respectively.[40] It has been stated that the activation of cell anti-apoptotic defense, which prevents the translation of drug-induced damage into cell death, is a key factor in cell anti-apoptotic resistance that decreases the cytotoxic effects of anticancer drugs. In this sense, a synthetic BH3 peptide was used in this study as a suppressor of cell anti-apoptotic defense. To clearly see the added therapeutic value when BH3 peptide was used, human ovarian carcinoma cells were incubated with free CPT, CPT-PEG, CPT-PEG-BH3, or CPT-PEG-LHRH conjugates and with the mixture of CPT-PEG-BH3 and CPT-PEG-LHRH conjugates. It was demonstrated that the pro-apoptotic activity of CPT was increased through a conjugation of CPT to PEG and that further enhancement was achieved by using the BH3 peptide in CPT-PEG-BH3 conjugate and LHRH peptide in CPT-PEG-LHRH conjugate and the mixture of both conjugates.[40,65]

Combination therapy has been also used to target tumor cells by oxidative stress. One example of this approach is that reported by Fang et al[59] for targeting the tumor compartment by oxidative damage using the combined actions of PEG-D-amino acid oxidase (DAO) conjugate and PEG-ZnPP.[59] PEG-DAO selectively delivers oxidative damage to the tumors, and PEG-ZnPP acts as a sensitizer, making cells more susceptible to these oxidative insults. This has been shown to be a powerful therapy, with a continuous suppression of tumor growth, even more than 20 days after the last treatment with PEG-DAO and

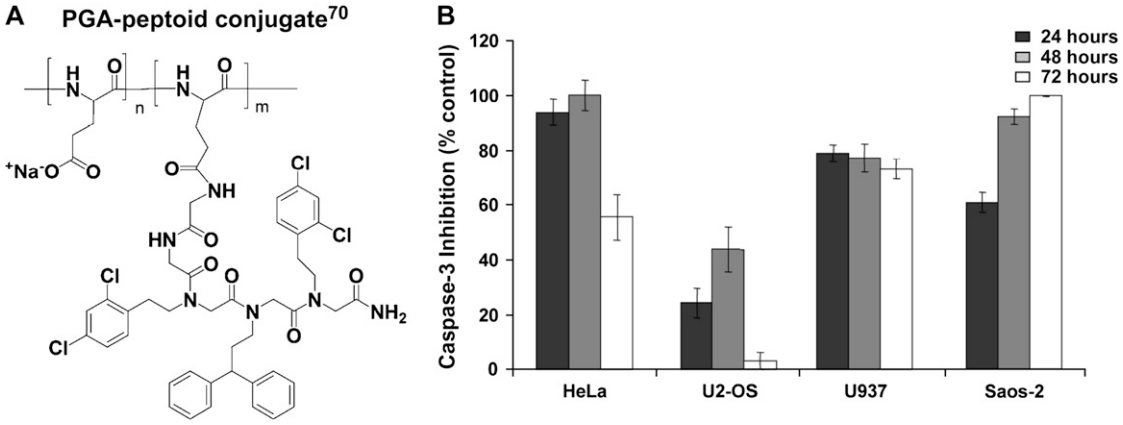

Figure 4. (a) Structure of PGA-peptoid conjugate as representative example of anti-apoptotic polymer-drug conjugates. (b) PGA-peptoid reduces induced apoptosis in different cell lines (Saos-2, U937, U2-OS, and HeLa human cell lines). Evaluation by caspase-3 activity in cell extracts measured by the fluorimetric DEVDase assay at 24-, 48-, and 72-hour incubation times, PGA-peptoid at 50-μM drug equivalent.[70] PGA indicates poly-L-glutamic acid and; DEVD, tetrapeptide aspartic acid-glutamine-valine-aspartic acid.

D-proline, and complete tumor regressions were observed in 3 of 8 treated mice.

Anti-Apoptotic Polymer-Drug Conjugates: Ischemia as a Target

Using apoptosis as a molecular target to prevent cell death is a much less explored approach. There are only 2 groups working within this context with clear therapeutic applications toward ischemic diseases.

Novel dendrimeric PEG conjugates releasing NO, PEG-NO conjugates, have been recently described by Pasut et al.[66] These conjugates were shown to inhibit arteriolar vasoconstriction and decrease oxidative stress significantly in an ischemia reperfusion model in vitro. Additionally, the conjugates also reduced the plasma von Willebrand level, lipid peroxides, and leukocyte adhesion on postcapillary venules during the ischemia-reperfusion injury. Consequently, PEG-NO conjugates could be a promising compound against oxidative stress in ischemia reperfusion injuries.[66]

Within the same context, our group in Valencia, Spain, has developed the first anti-apoptotic polymeric nanomedicine, PGA-peptoid conjugate, by the conjugation of a novel Apaf-1 inhibitor (peptoid 1) to PGA. As already mentioned in the introduction, apoptosome is a holoenzyme multiprotein complex formed by cytochrome c-activated Apaf-1, dATP, and procaspase-9 that links mitochondria dysfunction with the activation of the effector caspases. In turn, it is of interest for the development of apoptotic modulators.[67,68] Earlier we had developed a new structural class of Apaf-1 ligands as apoptosome inhibitors. The most potent of this family of N-alkylglycine inhibitors was peptoid 1.[69] However, this leading compound exhibited low membrane permeability and a modest efficiency, arresting apoptosis in cellular models. It was hypothesized that the conjugation of peptoid 1 to a polymeric carrier could offer a more specific intracellular trafficking that, together with an efficient lysosomotrophic drug release on the cytosol, would highly enhance its anti-apoptotic activity. PGA-peptoid conjugate clearly enhances the anti-apoptotic activity of peptoid 1 and diminishes its cytotoxicity in different cell models (Figure 4).[70] Preliminary studies of potential therapeutic interest with our apoptosome inhibitor showed certain biological activity as the apoptotic signs of cardiomyocytes decreased and were subjected to hypoxic conditions as a model of myocardial infarction.

Conclusion

Polymer-drug conjugates have enormous potential for researchers and clinicians. The great versatility of these macromolecular drugs enables the design and development of effective treatments for a variety of human pathologies. From macromolecular prodrugs of established anticancer agents, the applications of polymer-drug conjugates have expanded dramatically in recent years. Delivery of new anticancer agents,[71] combination therapy,[9,64,72-76] novel polymer architectures,[77] treatment of diseases other than cancer, and the use of molecular targets such as cellular apoptosis are the most exciting and promising areas.[7,78] Apoptosis as a molecular target allows the design of second-generation polymer conjugates for the treatment of a wide variety of human pathologies, from diabetes, heart failure, and brain stroke to diseases such as cancer. The pro-apoptotic applications have been extensively explored. However, the possibility of inhibiting the excess of cell death, at least in an acute form, is just beginning to be explored. It is hoped that in the near future some of these new approaches will reach clinical evaluation.

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