Poly-L-glutamic acid (PGA) Aided Inhibitors of Apoptotic Protease Activating Factor 1 (Apaf-1): An Antiapoptotic Polymeric Nanomedicine

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Abstract: An antiapoptotic polymeric nanomedicine, PGA−peptoid 1, has been developed by the conjugation of a novel apoptotic protease activating factor 1 (Apaf-1) inhibitor (peptoid 1) to poly-L-glutamic acid (PGA). Full structural and biophysical characterization of the conjugate by different techniques has been accomplished. This macromolecule clearly enhances the antiapoptotic activity of peptoid 1 in different cell models.

To accomplish the full therapeutic potential of a bioactive agent, it is crucial that it is delivered to the diseased area. Nanoscience and nanotechnology are innovative delivery techniques that offer not only potential benefits to patients but also new markets to the pharmaceutical industry. Specifically, it is fair to state that polymer therapeutics can be viewed as the first polymeric nanomedicines. These new chemical entities are nanosized hybrid constructs that covalently combine a bioactive agent with a polymer[1] to ensure not only its efficient release to the required intracellular compartment but also its availability within a specific period of time. Herein, we have developed a novel antiapoptotic polymer conjugate nanomedicine that has been demonstrated to have therapeutic potential as a targeted drug delivery system that offers great capabilities as an inhibitor of apoptosis.

Apoptosis, or programmed cell death, is a key cellular mechanism involved in a broad range of physiological processes. Deregulated apoptosis is associated with several human pathologies, such as cancer, ischemic injuries, and neurological disorders. The apoptotic cascade can be triggered through two major pathways.[2] Extracellular signals activate the receptor-mediated extrinsic pathway, while DNA damage, hypoxia, and other cell stress signals trigger the mitochondrial-dependent pathway where the roles of apoptotic protease activating factor 1 (Apaf-1) and apoptosome activation have been well established. The apoptosome is a holoenzyme multiprotein complex formed by cytochrome *c* activated Apaf-1, dATP, and procaspase-9.[3] Therefore, inhibition of the apoptosome assembly represents an interesting target for the development of apoptosis modulators.[4] We previously developed a new structural class of Apaf-1 ligands as apoptosome inhibitors. From this family of *N*-alquilglycine inhibitors, the most potent in vitro was peptoid 1 (**1**).[5] However, this lead compound exhibited low membrane permeability and modest efficiency, arresting apoptosis in cellular models. It was hypothesized that the conjugation of **1** to a polymeric carrier could offer a more specific intracellular trafficking that coupled to an efficient lysosomotropic drug release on the cytosol would highly enhance the antiapoptotic activity of peptoid 1.

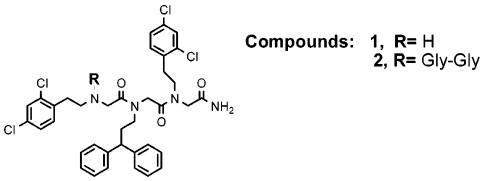
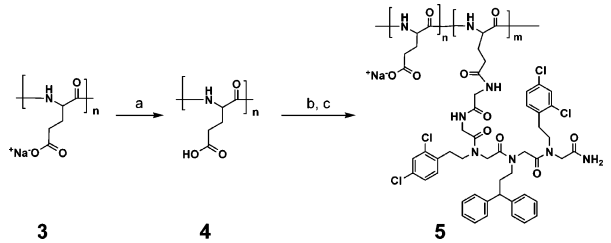


Figure 1. Structure of peptoid 1 (**1**) and GG−peptoid 1 (**2**).

Scheme 1. Synthesis of PGA-peptoid Conjugate **5**[a]



[a] Reagents and reaction conditions: (a) 2 N HCl, pH 2, 90%; (b) DIC, HOBt, DIEA, anhydrous DMF, **2**, room temp, 36 h, two steps; (c) $NaHCO_3$, pH 8, 92%.

The aim of this study therefore was to design, synthesize, and characterize a polymer conjugate that, by carrying a novel Apaf-1 inhibitor, would rescue cells from inappropriate apoptosis. Poly-L-glutamic acid (PGA) (**3**) was chosen as a polymeric carrier because it can be readily degraded by lysosomal enzymes, it is stable in plasma, and it contains functional groups for drug attachment. In addition, PGA has excellent pharmacological properties, as demonstrated by Xyotax,[6,7] which could represent the first polymer−anticancer drug conjugate to be commercialized.

The PGA−peptoid 1 conjugate (**5**) was synthesized using a DIC-mediated coupling through a diglycil spacer (using GG−peptoid 1 as a reagent (**2**), Figure 1) to contain peptoid 1 up to a 12 mol % loading (free drug content less than 1 wt % of total drug), as determined by UV−vis spectrophotometry and HPLC analysis (Scheme 1 and Supporting Information Figure 1S).

The average molecular weight (M_w), polydispersity (M_w/M_n), and the behavior of both PGA−peptoid **5** and control PGA in solution were analyzed by size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC). Both techniques showed PGA−peptoid as a more compact conformational structure with a smaller hydrodynamic volume and a greater sedimentation coefficient (S) than PGA ($t_r = 12.8$ min and $S = 3.5 \pm 0.4$ s vs $t_r = 11.6$ min and $S = 1.9 \pm 0.1$ s for **5** and **3**, respectively). By sedimentation equilibrium, M_w was determined as 26 700 Da for **3** and 60 300 Da for **5**. However, M_w of 35 000 Da ($M_w/M_n = 1.8$) and 29 000 Da ($M_w/M_n = 1.6$) were determined for **3** and **5**, respectively, by SEC using PEG standards. This correlates with previous studies that showed how polymer−drug conjugates exist in solution as unimolecular micelles and also how conformation is influenced by drug loading and drug nature.[8] The hydrophilicity of PGA and hydrophobicity of peptoid 1 favor the formation of compact unimolecular micelles.

NMR spectroscopy confirmed the integrity of peptoid 1 and its covalent attachment to PGA (**3**). The 1D ^1H NMR spectrum of PGA−peptoid **5** showed extra resonances arising from the peptoid moiety plus a general broadening of the signals when

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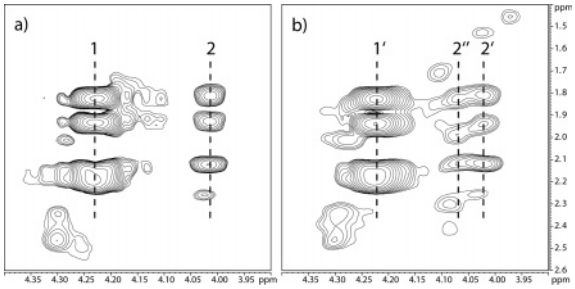

Figure 2. Expansion of 2D TOCSY NMR spectra of PGA (**3**) (a) vs PGA−peptoid **5** (b).

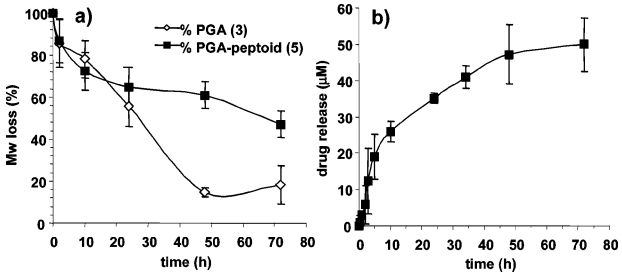

Figure 3. Cathepsin B degradation study: (a) percentage of molecular weight (M_w) loss of PGA−peptoid and PGA, measured by SEC; (b) release profile of peptoid 1 from PGA−peptoid **5**, determined by HPLC.

compared to the spectrum from PGA (Supporting Information Figure 2S). The most affected signals are those corresponding to the NH-amide protons (at 8.5 ppm), changing from sharp *J*-coupled resonances in the PGA spectrum (unstructured polymer) to broad signals in the PGA−peptoid spectrum, as a consequence of both the NH averaging of functionalized and nonfunctionalized side chains, and conformational changes. In addition, the 2D NOESY spectrum of **5** showed cross-peaks correlating the aromatic groups from **1** (6.7−7.4 ppm) with the glutamic acid side chain (1.8−2.2 ppm) and Hα (4.0−4.3 ppm) from the polymer. Furthermore, whereas the 2D TOCSY spectrum of **3** showed two glutamic acid Hα signals (corresponding to polymer main chain (1) and termini (2)) that correlate with the glutamic side chain protons, the PGA−peptoid TOCSY spectrum showed an additional Hα connected to a new glutamic side chain arising from functionalized termini (2″). Therefore, drug−polymer connectivity is confirmed (Figure 2 and Supporting Information Figure 3S).

Effective biological activity of polymer−drug conjugates relies on (i) stability in blood circulation and (ii) lysosomal enzyme cleavage to release active drug in a specific place after cellular uptake by endocytosis. PGA−peptoid **5** was completely stable in both plasma and buffers but was degraded in the presence of the lysosomal enzymes (cathepsin B) releasing the drug in a time-dependent manner (Figure 3). Peptoid 1 release began after enzyme addition (Figure 3b), reaching a plateau after 50 h. The lowered rate of degradation when compared to **3** is in agreement with the more compact conformation observed by AUC that could limit enzyme accessibility. Gly−peptoid 1 was the major degradation compound obtained (as determined by HPLC and MALDI-TOF) and showed in vitro activity similar to that of the parent drug (Supporting Information Figure 4S).

After full chemical characterization, **5** was evaluated in different human cell models of intrinsic (U937 histiocytic lymphoma, HeLa adenocarcinoma and U2OS osteosarcoma cells challenged with doxorubicin (Dox), and Saos-2 osteosarcoma cells with the conditional expression of the proapoptotic protein Bax (Saos Bax tet-ON)) and extrinsic apoptosis (mouse embryo fibroblasts (MEFs) challenged with TNF-α). The PGA−peptoid did not show unspecific cell toxicity (up 100 μM drug equiv). Dox induces apoptosis through DNA damage that is transduced to the mitochondria by disturbing the mitochondrial membrane potential (MMP) and by activating executioner caspases through the involvement of apoptosome. In these models, PGA−peptoid markedly reduced Dox-induced apoptosis (up to 90%) in a concentration- and time-dependent manner (see Supporting Information Figure 5S and Table 1S). Although Dox-induced apoptosis has been used in different studies, Dox was found to induce intrinsic and extrinsic forms of apoptosis

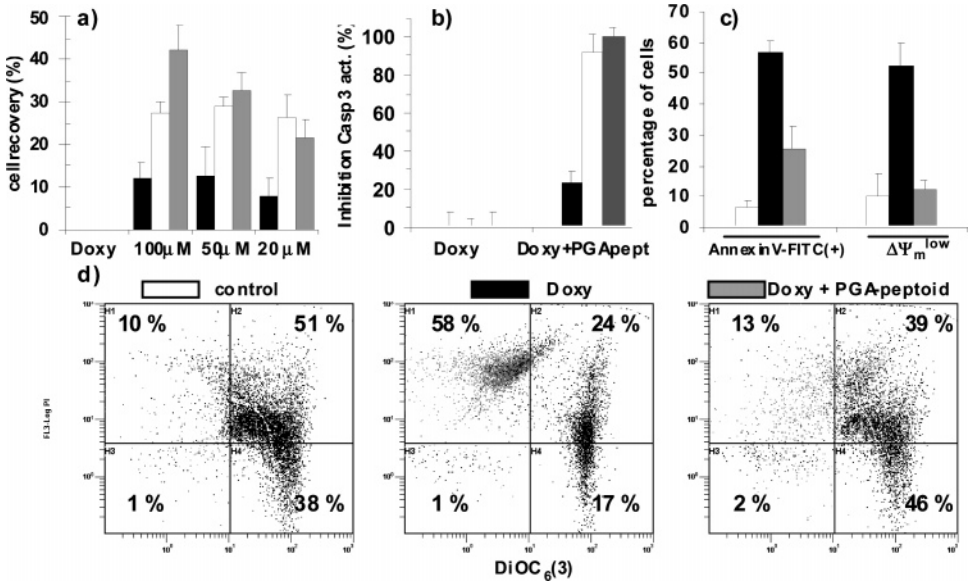

Figure 4. PGA−peptoid **5** reduces Bax-induced apoptosis in Saos-2 cells, with cells cultured in the absence (control) or presence of 2 μM Doxy or Doxy plus PGA−peptoid (Doxy + PGApeptoid): (a) evaluation by MTT assay of cell death inhibition by **5** at different concentrations (*x* axis) and incubation times (24, 48, and 72 h (black, white, and gray bars, respectively)); (b) caspase 3 activity in cell extracts measured by the fluorimetric DEVDase assay at 24, 48, and 72 h incubation times (black, white, and gray bars, respectively), **5** at 50 μM drug equiv; (c) evaluation of apoptosis by flow cytometry as the percentage of cells with exposed phosphatidylserine (annexinV−FITC positive) and low $\Delta\Psi_m$ ($\Delta\Psi_m^{low}$); (d) flow cytometric analysis of $\Delta\Psi_m$ of cells treated after 72 h of incubation and **5** at 50 μM drug equiv. Numbers refer to the percentages of cells in the different regions. Data are expressed as the mean ± SD ($n > 3$).

through mitochondria-derived ROS and the modification of cell calcium homeostasis.[9] Therefore, we used Saos-2 cells to obtain a more detailed analysis in an "only intrinsic form" of apoptosis. In this model, conjugate **5** also showed a dose- and time-dependent prevention of cell-viability loss, induced by the expression of Bax as measured by MTT assays (Figure 4a) and by the inhibition of caspase-3 activity (Figure 4b), which is consistent with the inhibition of Apaf-1 dependent apoptosis. Additionally, doxycycline (Doxy) treated cells demonstrated staining for annexin V-FITC (that specifically binds to exposed phosphatidylserine) and loss of MMP, as determined by using $DiOC_6(3)$. Doxy-treated cells in the presence of **5** showed a diminished Doxy-induced apoptotic phenotype (Figure 4c,d). Finally, it was also proved that PGA−peptoid did not inhibit TNF-α induced extrinsic apoptosis, suggesting that only the mitochondrial pathway of caspase activation is inhibited.

In short, we have developed the first antiapoptotic polymeric nanomedicine that demonstrates that the conjugation of the novel Apaf-1 inhibitor peptoid 1 to PGA clearly enhances its anti-apoptotic activity and diminishes its cytotoxicity in different cell models. Apoptosis is a relevant cell death mechanism in the pathogenesis of acute and chronic cardiodegenerative and neurodegenerative disorders. Thus, an increasing number of compounds with the potential to inhibit apoptosis are being explored at preclinical and clinical levels.[10] In particular, the use of caspase inhibitors in preclinical animal models reduces the ischemic area size and improves the acute functional parameters.[11,12] PGA−peptoid inhibits the activity of the apoptosome and could help to keep caspases silent, or it would be useful in combined applications with inhibitors of caspases. Additionally, this polymer conjugate also provides the opportunity to introduce specific residues for active disease-targeting.[13,14] This fact would increase the range of therapeutic opportunities for apoptosis treatment.

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Supporting Information Available: Full experimental details and complementary information. This material is available free of charge via the Internet at http://pubs.acs.org.

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