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Intracellular Delivery of the Reactive Oxygen Species Generating Agent D-Penicillamine upon Conjugation to Poly-L-glutamic Acid

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Abstract: D-Penicillamine is an aminothiol that is cytotoxic to cancer cells and generates dose dependent reactive oxygen species (ROS) via copper catalyzed oxidation. However, the delivery of D-pen to cancer cells remains a challenge due to its high hydrophilicity, highly reactive thiol group and impermeability to the cell membrane. To overcome this challenge, we investigated a novel poly-L-glutamic acid (PGA) conjugate of D-pen (PGA−D-pen) where D-pen was conjugated to PGA modified with 2-(2-pyridyldithio)-ethylamine (PDE) via disulfide bonds. Confocal microscopy and cell uptake studies showed that the fluorescently labeled PGA−D-pen was taken up by human leukemia cells (HL-60) in a time dependent manner. Treatment of HL-60, murine leukemia cells (P388) and human breast cancer cells (MDA-MB-468) with PGA−D-pen resulted in dose dependent cytotoxicity and elevation of intracellular ROS levels. PGA−D-pen induced apoptosis in HL-60 cells which was verified by Annexin V binding. The *in vivo* evaluation of the conjugate in the P388 murine leukemia model (intraperitoneal) resulted in significant enhancement in the survival of CD2F1 mice over vehicle control.

Keywords: Apoptosis; thiol; leukemia; polymer; copper

Introduction

Cancer cells are under persistent increased reactive oxygen species (ROS) stress due to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction.[1] Such a sustained oxidative stress and thus exhausted redox buffering capacity makes cancer cells more susceptible to killing by oxidative insult compared to normal cells.[2] Therefore, agents that elevate intracellular ROS levels can lead to cytotoxic effects in cancer cells.

D-Penicillamine (D-pen) is an aminothiol and a strong copper chelator. It has been registered by the FDA for the treatment of Wilson's disease. We recently showed that D-pen is cytotoxic to leukemia (HL-60, HL-60/VCR) and breast cancer cells (MCF-7 and BT-474) when externally supplied with copper via concentration dependent generation of hydrogen peroxide (H_2O_2).[3,4] The mechanism of H_2O_2 generation in the presence of copper was first proposed by

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Starkebaum and Root.[5] In a recent study, D-pen was found to produce maximum amount of ROS among different amino thiols due to favorable pK_a (7.9) of its thiol group.[6] The H_2O_2 generated by D-pen oxidation may result in a ROS cascade involving hydroxyl radical via Fenton type reactions.[7]

Copper has been established as a key cofactor required by a number of proangiogenic molecules including fibroblast growth factor, vascular endothelial growth factor and interleukin-1.[8,9] Several *in vitro* studies have shown that high copper concentrations facilitate proliferation of cancer cells.[10−12] Significantly elevated levels of copper have been found in the serum and tumors of patients compared to healthy individuals.[13−17] Therefore, the delivery of D-pen to the cancer cells may result in a dual anticancer effect involving metal catalyzed elevation of cellular ROS levels leading to cytotoxicity (mechanism 1) as well as an antiangiogenic effect (mechanism 2). In fact, D-pen has been explored in the clinic as an antiangiogenic agent. For example, Matsubara et al. reported that D-pen inhibited human endothelial cell proliferation and endothelial cell growth factor induced neovascularization in rabbit cornea.[18]

Although a few previous studies have shown D-pen to cause cytotoxicity in the presence of copper in *in vitro* studies, these effects were almost certainly due to the extracellular production of cytotoxic H_2O_2[19−22] since our recent studies confirmed that D-pen is impermeable to cancer cells.[20] Thus, the cell membrane presents a significant barrier to the therapeutic delivery of D-pen as a ROS-producing cytotoxic agent. Moreover, D-pen has been shown to rapidly oxidize to D-pen disulfide *in vivo* and bind strongly to the plasma proteins, mainly albumin, via thiol disulfide exchange.[21,22] This further limits the availability of D-pen for uptake by the cancer cells. Thus, there is a need to devise a delivery system for D-pen that could (i) protect the thiol group; (ii) enhance the intracellular delivery; and (iii) deliver high concentration of D-pen to cancer cells. It is hypothesized that conjugation of D-pen to the pendant groups of a polymer via disulfide bonds could lead to the delivery of higher concentrations of D-pen resulting in enhanced intracellular accumulation of D-pen by uptake via endocytosis. It is further hypothesized that release of D-pen from its polymeric conjugate in the intracellular reducing environment will result in time and concentration dependent cytotoxicity in cancer cells via the generation of cytotoxic ROS.

Polymer−drug conjugates or "polymer therapeutics"[19] provide distinct advantages for the delivery of small drug molecules by acting as passive targeting carriers via enhanced

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uptake and longer retention in the tumors. This ability has been attributed to the characteristic leaky vasculature of tumors termed as the "enhanced permeability and retention" (EPR) effect by Maeda.[23,24] Several polymer−drug conjugates are being investigated for their potential to enhance the anticancer efficacy of drugs bound to them.[25]

Our previous studies using a gelatin−D-pen conjugate showed cellular uptake and cytotoxicity in leukemia cells.[20] However, the conjugation efficiency was low and the conjugate only showed long-term cytotoxicity possibly due to slower uptake and release of D-pen from the conjugate. Poly-L-glutamic acid (PGA) is a biodegradable and biocompatible polymer composed of L-glutamic acid monomer units linked together with amide bonds. The pendant carboxyl groups of PGA provide excellent sites for drug conjugation. Several anticancer drugs have been conjugated to PGA via ester,[26−28] hydrazone[29] or amide bonds,[30] with or without spacers[31,32] between the drug and the polymer.

In the present studies, we describe the synthesis, characterization and *in vivo* anticancer activity of a novel water-soluble PGA−D-pen conjugate where D-pen is covalently bound to PGA via a disulfide bond. The conjugate was also investigated for its ability to enhance the intracellular delivery of D-pen and subsequent cytotoxicity through a ROS-mediated mechanism in leukemia cells.

Experimental Section

Materials. Poly-L-glutamic acid (MW 50−70 kDa), D-penicillamine (D-pen), D-penicillamine disulfide, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), DL-dithiothreitol (DTT), sodium borohydride ($NaBH_4$), Sephadex G-25 medium, chloroquine diphosphate and ammonium dihydrogen phosphate were purchased from Sigma-Aldrich (St. Louis, MO). NHS−fluorescein and BCA protein assay kit was purchased from Pierce Biotech Inc. (Rockford, IL). Acetonitrile, *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO) and *o*-phosphoric acid (85%) were purchased from Fisher Scientific (Pittsburgh, PA). Carboxy-H_2DCFDA and propidium iodide were purchased from Invitrogen (Carlsbad, CA). Annexin-V-FITC was purchased from BD Pharmingen (San Diego, CA).

Synthesis of PGA−D-Pen Conjugate. PGA−D-pen conjugate was synthesized as shown in Scheme 1. 2-(2-Pyridyldithio)ethylamine (PDE) hydrochloride was synthesized according to a previously published method.[33] PDE was covalently conjugated to PGA via an amide bond. A large molar excess of D-pen was used to conjugate it to the modified PGA via thiol−disulfide exchange. Briefly, PGA (100 mg, 0.63 mmol of carboxy monomer), PDE hydrochloride (279.78 mg, 1.26 mmol), *N*-hydroxysuccinimide (NHS) (36.15 mg, 0.314 mmol) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (120.45 mg, 0.63 mmol) were added to 5 mL of DMF. Triethylamine (1.5 mmol) was added to the reaction. The mixture was then stirred for 12 h at room temperature under nitrogen. After the reaction, the solvent was removed by vacuum evaporation, and the product washed three times with dichloromethane to remove excess reactants. The product was then resuspended in 0.05 M borate buffer pH 9.0, and D-pen (234.4 mg, 1.26 mmol) was added. The mixture was stirred for 12 h at room temperature. PGA−D-pen was purified and exchanged with PBS pH 7.4 using a Sephadex G-25 column (1.5 × 30 cm).

To synthesize fluorescently labeled PGA−D-pen conjugate, 0.04 mL of NHS−fluorescein in DMSO (3.2 mM) was added to 0.45 mL of PGA−D-pen conjugate in PBS buffer pH 7.4. The reaction mixture was stirred in the dark for 1 h at room temperature. The fluorescently labeled conjugate was purified using a Sephadex G-25 column (1.5 × 30 cm). The moles of fluor per mole of PGA were determined spectrophotometrically (ε=68000 M^{-1} cm^{-1}, λ_{max}= 494 nm).

Choice of Reducing Agent to Measure the Extent of Conjugation. Three different reducing agents, sodium borohydride ($NaBH_4$), DL-dithiothreitol (DTT) and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were screened. Briefly, PGA−D-pen conjugate was incubated with increasing

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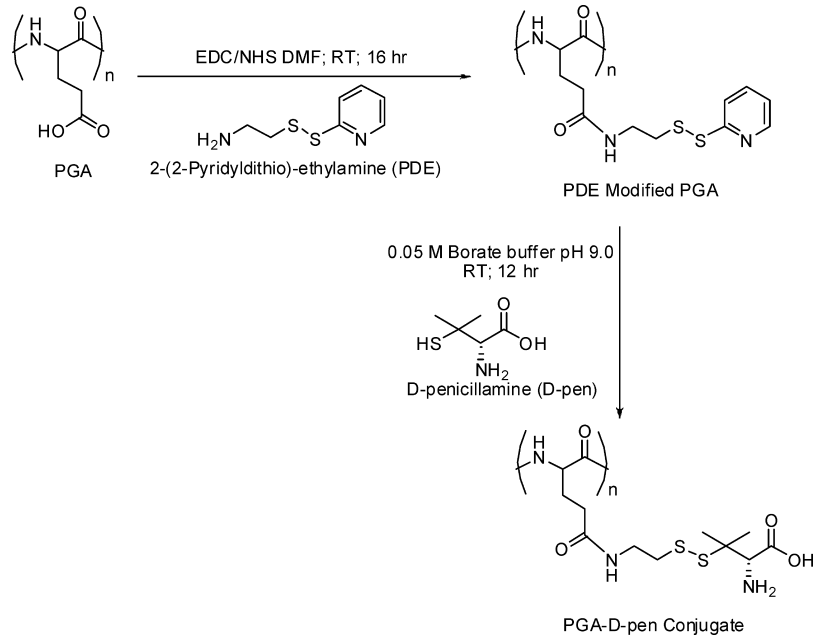
Scheme 1. Synthesis of PGA−D-Pen Conjugate[a]



[a] PGA was covalently linked to PDE using EDC/NHS chemistry. D-Pen was conjugated to modified PGA via thiol−disulfide exchange of pyridine-2-thione with D-pen at pH 9.0. Abbreviations: PGA, poly-L-glutamic acid; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide.

concentration (5 mM to 25 mM) of the reducing agent for varying time (30 min to 4 h) and the amount of D-pen released was determined using HPLC. Based on the results, TCEP was chosen for further studies.

Quantification of D-pen Conjugation by HPLC. 100 mM TCEP (0.25 mL) was added to PGA−D-pen conjugate (0.75 mL) and stirred for 1 h at room temperature. D-pen released upon reduction of the PGA−D-pen conjugate was analyzed with a modification of our previously reported HPLC method.[4] The HPLC analysis was preformed using a Finnigan™ Surveyor HPLC System (Thermo Electron Corp., San Jose, CA) with a Gemini C18 column (250 × 4.6 mm; 5 μM; 20 μL sample; Phenomenex, Torrance, CA). The mobile phase employed was 20 mM ammonium dihydrogen phosphate +3% acetonitrile adjusted to pH 2.5 using o-phosphoric acid, and pumped at a flow rate of 1 mL/min. D-pen and D-pen disulfide were detected by UV absorption at 214 nm with retention times of 5.2 and 4.8 min, respectively.

Cell Uptake Studies. The uptake of the fluorescently labeled PGA−D-pen conjugate was determined qualitatively using confocal microscopy. HL-60 cells (5×10^5) cultured in RPMI-1640 without phenol red and supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin, were plated in a 24 well plate and treated with fluorescently labeled PGA−D-pen conjugate (500 μM D-pen equivalent). Cells were washed with PBS and resuspended in RPMI 1640 without phenol red and immediately observed under a confocal microscope. Cells were transferred onto a slide for visualizing using Zeiss 510 Meta Laser Scanning Confocal Microscope (63 × 1.4 NA oil Plan-Apochromat objective; excitation =488 nm and emission =515 nm; Carl Zeiss, Thornwood, NY). Differential Interference Contrast (DIC) images, fluorescence images and the overlapped images taken from the microscope were visualized using the Zeiss AIM Viewer (Carl Zeiss, Thornwood, NY).

To quantitatively determine D-pen associated with HL-60 cells, 5×10^5 cells were incubated with PGA−D-pen (500 μM D-pen equivalent) for predetermined time points. The cells were centrifuged at 1000 rcf for 10 min and the supernatant was reduced with TCEP to release free D-pen which was analyzed by HPLC. This was subtracted from the original amount to obtain the cell-associated PGA−D-pen.

Cytotoxicity Studies in Cells. The HL-60 and MDA-MB-468 cells were obtained from American Type Cell Culture Collection (ATCC; Rockville, MD). The P388 cells were obtained from National Cancer Institute-Frederick Cancer Research Facility, DCT Tumor Repository (NCI, Bethesda, MD). HL-60 and P388 cells were cultured in RPMI-1640 (Invitrogen) while MDA-MB-468 cells were cultured in DMEM (Invitrogen). The media were supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% Fetal Bovine Serum (FBS) (ATCC). All cell lines were maintained at 37 °C in a humidified 5% CO_2 incubator. Cell viability was regularly determined by trypan blue dye (0.4% in phosphate buffered saline) (ATCC).The P388 cells and HL-60 cells were plated at 1×10^4 and 4×10^4 cells respectively in 200 μL of medium per well in round-bottom 96-well microwell plates. The MDA-MB-468 cells were plated at 1×10^4 cells/well in 96-well flat bottom microwell plates and allowed to attach overnight. Equal volumes of PGA−D-pen conjugate, PGA, D-pen, PGA+D-pen or PBS were added and the plates were incubated for 48 h (37 °C, 5% CO_2). Twenty (20) μL of MTT reagent (5 mg/mL in PBS) was added to each well and reincubated for 3−4 h to allow formation of

formazan crystals. The round-bottom plates were centrifuged at 200 rcf for 5 min. Subsequently, the supernatant was aspirated and 200 μL DMSO was added to each well and the plate was incubated at room temperature for 1 h to lyse the cells and solubilize formazan. The optical density of each well at 570 nm was measured on a Synergy™ 2 Multi-Detection Microplate Reader (Biotek; Winooski, VT). The results were analyzed in terms of percentage of viable cells after 48 h of incubation as compared to control cells.

Intracellular ROS Generation. ROS generation was assessed using 5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) (Molecular Probes). Carboxy-H_2DCFDA is a cell permeant probe and has improved intracellular retention due to additional negative charges at cytosolic pH. It is converted to a highly fluorescent form upon deacetylation by cellular esterases and oxidation. Stock solutions of carboxy-H_2DCFDA (2 mM) were prepared in DMSO. Further dilutions were prepared in PBS. For ROS measurement, HL-60 cells were incubated for 30 min in PBS containing 25 μM carboxy-H_2DCFDA. Subsequently, the cells were washed with PBS and resuspended in RPMI-1640 without phenol red and serum. Three $\times 10^4$ cells were plated in 96-well dark flat bottom plates and treated with different concentrations of D-pen and PGA−D-pen conjugate. Fluorescence was determined at various time points post-treatment using Synergy™ 2 Multi-Detection Microplate Reader at excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 30 nm. RPMI-1640 and cells not incubated with the probe were used as negative controls while 100 μM H_2O_2 was used as positive control.

Apoptosis Assay. HL-60 cells (5×10^5) were incubated with PGA−D-pen (100−1000 μM D-pen equivalent) for 2 h, 6 h, 14 h and 24 h followed by double staining with Annexin-V-FITC and propidium iodide (PI) to differentiate between live, necrotic and apoptotic cells. Briefly, the cells were centrifuged, washed with PBS and resuspended in 0.1 mL Annexin-V binding buffer. Annexin-V-FITC (5 μL) was added and incubated for 15 min in dark at room temperature. PI (10 μL of 50 μg/mL stock solution in binding buffer) and 0.4 mL of binding buffer were added and cells were immediately analyzed by flow cytometry (Becton-Dickinson). Untreated HL-60 cells (negative control), cells treated with 10 μM etoposide (Annexin-V-FITC positive control) and cells treated with ethanol for 20 min (PI positive control) were analyzed to adjust instrument compensation and setting up the quadrant statistics. The results were processed by Cellquest (Becton-Dickinson) and FlowJo (Tree Star).

In-vivo Anticancer Efficacy. To determine the acute toxicity, PGA−D-pen conjugate in 0.9% sodium chloride (300 mOsmol, pH 7.4, sterile filtered) was administered ip to CD2F1 mice (16−18 g) at doses of 1, 2, 5, and 10 mg/kg D-pen equivalent on day 1, 5, and 9. Sodium chloride (0.9%) was administered ip as vehicle control to untreated animals. The mice were monitored for 14 days for mortality and were assigned a body condition score (BCS)[34] every 48 h based on body weight and general health criteria. A loss of 10% body weight over 3 days or loss of 20% of initial weight and a BCS score of less than 2 was considered to be criteria for euthanasia.

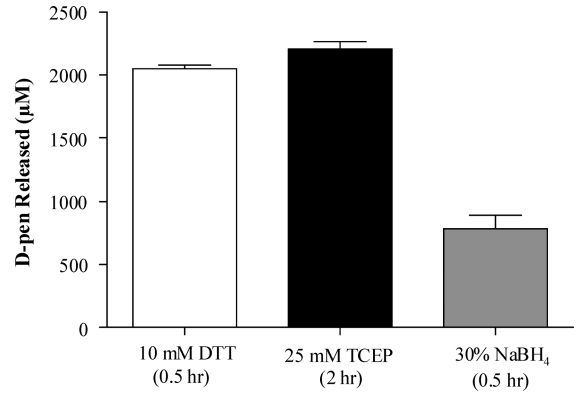


Figure 1. **Screening of disulfide reducing agents.** PGA−D-pen was incubated separately with different concentrations of three reducing agents for predetermined time and released free D-pen was analyzed by HPLC. Each bar represents mean ± SD (n = 3) of the concentration and time at which the highest amount of D-pen was released.

Anticancer efficacy of the PGA−D-pen conjugate was assessed using the intraperitoneal P388 murine leukemia model. Briefly, 1×10^5 P388 murine leukemia cells were implanted in the intraperitoneal (ip) cavity of CD2F1 mice (16−18 g) on day 0. PGA−D-pen was administered ip on day 1, 5, and 9 at 5 mg/kg and 10 mg/kg D-pen equivalent respectively. The mice were observed daily for mortality and were assigned a BCS every 48 h. The percent survival curves were plotted and median survival was determined. All experiments involving mice were performed with the approval of the University of North Carolina Institutional Animal Care and Use Committee.

Statistical Analysis. Statistical analysis was performed with GraphPad Prism 4 Software (GraphPad software Inc. San Diego, CA). Results were depicted as mean ± SD. IC_{50} values were derived from the percent viability data by non linear regression curve fitting. ROS generation was analyzed by one-way ANOVA followed by Dunnet's post test to compare the different dose levels to control. Survival curves from the anticancer efficacy studies were plotted by Kaplan−Meier's method and analyzed by Mantel-Cox log-rank test.

Results

Synthesis and Characterization of PGA−D-pen Conjugate. PGA−D-pen conjugate was synthesized by modifying PGA with PDE as shown in Scheme 1. PDE is a heterobifunctional cross-linker as the amine group can be used to conjugate with carboxy groups while the pyridyl thiol

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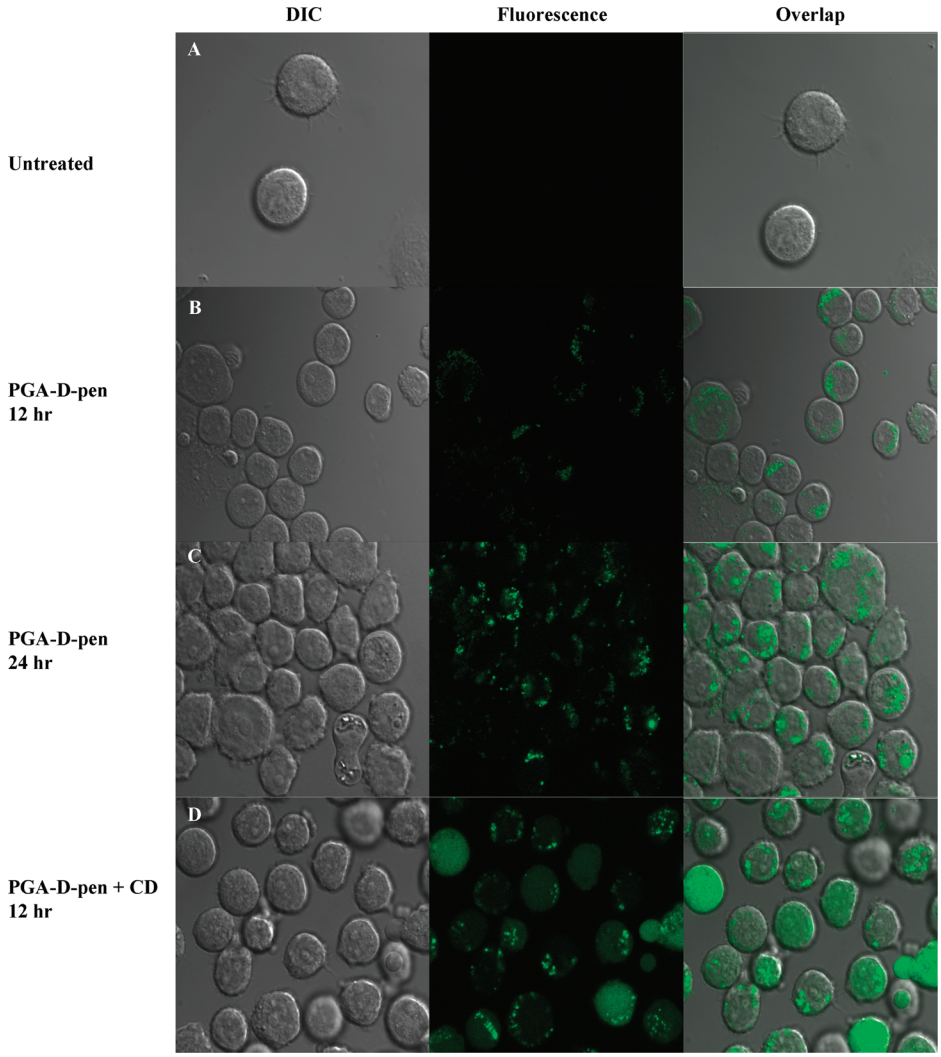

Figure 2. Intracellular uptake of PGA-D-pen conjugate by confocal microscopy. Differential interference contrast (DIC) images, fluorescence images and the overlapped images of untreated HL-60 cells (A), HL-60 cells treated with fluorescently labeled PGA-D-pen conjugate for 12 h (B), 24 h (C) and 12 h in the presence of chloroquine diphosphate (D).

functionality provides an opportunity to conjugate thiol groups via thiol-disulfide exchange in the presence of molar excess of another thiol like D-pen. The second conjugation reaction was performed in the presence of borate buffer pH 9.0 to provide favorable conditions for thiol-disulfide exchange (pK_a of D-pen thiol is 7.9). The final conjugate was completely soluble in water.

To determine the efficiency of conjugation, different disulfide reducing agents were screened. Among the agents tested, TCEP was selected for further studies as it was the most efficient reducing agent and did not interfere with the HPLC assay (Figure 1). The previously developed HPLC assay[4] was modified to determine D-pen, D-pen disulfide and TCEP before and after reduction. The final conjugate had 35 moles of D-pen per mole of PGA. The theoretical maximum weight loading of D-pen on PGA was 8 wt % (9.3% of the pendant carboxyl groups were modified per chain of PGA). ^{1}H NMR (Inova 500 spectrometer; 500 MHz in D_2O) of the conjugate showed resonance of D-pen at 3.81 ppm (1 H, C2), 1.85 ppm (3 H, C3-methyl) and 1.68 ppm (3 H, C3-methyl), resonance of methylene protons of PDE at 3.03 ppm (2 H, CH_2-S) and 3.13 ppm (2 H, CH_2-N) and resonance of PGA at 4.20 ppm (1 H, $C_\alpha-H$), 2.16 ppm (2 H, C_γ-H_2) and 1.84 ppm (2 H, C_β-H_2).

Cell Uptake Studies. PGA−D-pen was fluorescently labeled using NHS−fluorescein. The number of moles of fluorescein per mole of PGA was 1.34, which was determined spectrophotometrically. The intracellular uptake was visualized by confocal microscopy. Live cells were visualized under the microscope after exposure to the conjugate for a predetermined time. The control cells were visualized to confirm that the cells were healthy and to rule out any background fluorescence. The conjugate exhibited time dependent uptake in HL-60 cells (Figure 2). To further confirm the results obtained by confocal microscopy, cell associated PGA−D-pen was determined by HPLC. Up to 25% PGA−D-pen was found to be associated with the cells at 8 h. These results support the hypothesis that polymer conjugation increases the cellular uptake of D-pen.

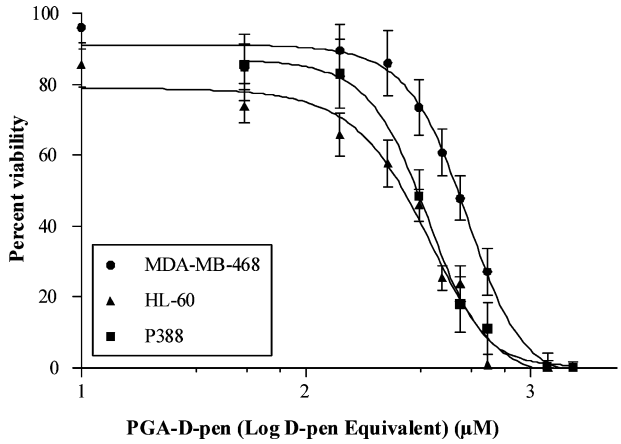

Figure 3. Cytotoxicity of PGA−D-pen conjugate in (a) HL-60 cells; (b) P388 cells and (c) MDA-MB-468 cells. Cytotoxicity was determined by MTT assay 48 h after treatment with PGA−D-pen. The log of equivalent D-pen concentration was plotted on the *X*-axis. Each point represents mean ± SD ($n = 3$).

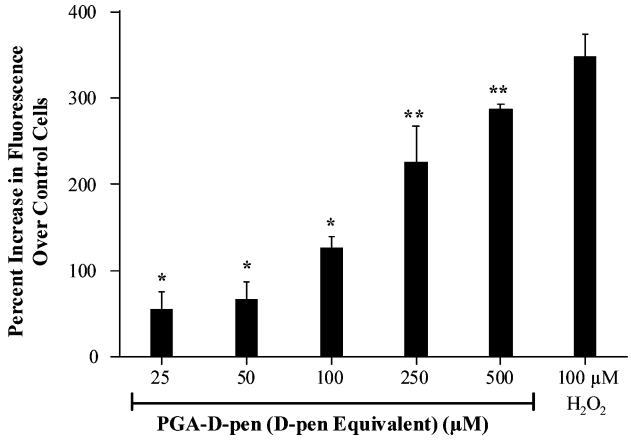

Figure 4. Intracellular ROS generation by PGA−D-pen conjugate in HL-60 cells. Cells were incubated with 25 μM carboxy-H_2DCFDA for 30 min before exposure to PGA−D-pen conjugate. H_2O_2 (100 μM) was used as positive control. The fluorescence values were measured postincubation at 8 h with the conjugate and at 30 min with H_2O_2 respectively. Each bar represents mean ± SD ($n = 3$). *$p < 0.01$ and **$p < 0.001$ compared to the control untreated cells.

Cytotoxicity Studies in Cells. The *in vitro* cytotoxicity of the conjugate was investigated in leukemia (HL-60 and P388) and human breast cancer cells (MDA-MB-468). The PGA−D-pen conjugate treatment resulted in a dose-dependent reduction in viability of the cells (Figure 3). The IC_{50} values for HL-60 (4×10^4 cells), P388 (1×10^4 cells) and MDA-MB-468 (1×10^4 cells) were 106.1 μM, 106.3 μM, 156.7 μM, respectively. Similar concentrations of free D-pen, PGA or D-pen + PGA did not cause significant reduction in cell viability over the duration of study. Our previous studies have shown that D-pen alone is cytotoxic to cancer cells only at low millimolar concentrations.[3]

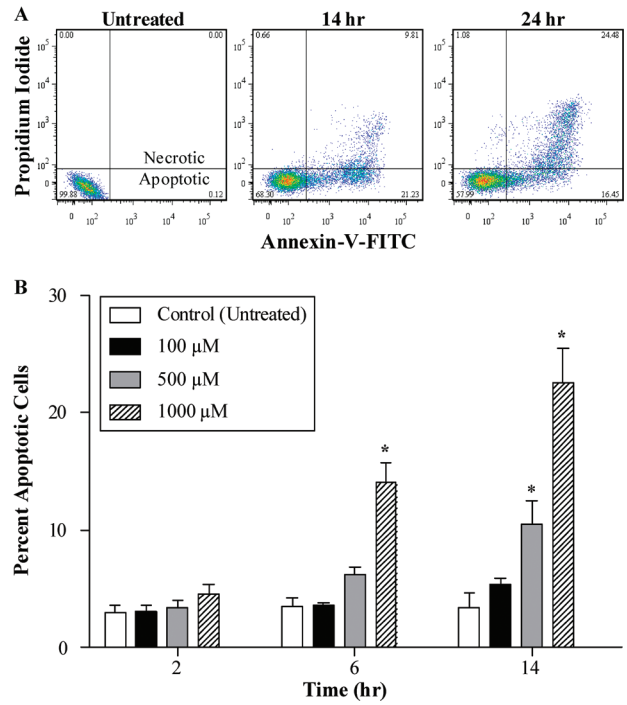

Figure 5. Apoptosis induction by PGA−D-pen. Human leukemia cells (HL-60; 5×10^5) were treated with three different concentrations of PGA−D-pen followed by double staining with Annexin-V−FITC/PI at different time points. A: Flow cytometric analysis of HL-60 cells treated with 1000 μM PGA−D-pen conjugate (D-pen equivalent). B: The percent apoptotic cells (FITC positive, PI negative) at different time points. Each bar represents mean ± SD ($n = 3$). *$p < 0.001$ compared to the control untreated cells.

Intracellular ROS Generation. Generation of ROS upon treatment with the conjugate was investigated using carboxy-H_2DCFDA, a nonfluorescent probe which gets converted to highly fluorescent derivative following deacetylation by intracellular esterases and oxidation. This dye was chosen due to its longer intracellular retention compared to H_2DCFDA used in our previous studies. The time and dye concentration required for the study were optimized using H_2O_2, which was also used as a positive control. The ROS levels were significantly higher compared to the control at all concentrations tested (Figure 4). Maximal levels of ROS were observed at 8 h after treatment with PGA−D-pen. The ROS levels at the highest concentration of the conjugate, i.e. 500 μM (D-pen equivalent), were not significantly different from the levels produced by 100 μM H_2O_2, which suggests the strong potential of the synthesized conjugate to generate intracellular ROS upon release of D-pen.

Apoptosis Induction. Phosphatidylserine (PS) is distributed asymmetrically in the plasma membrane of live cells and inverts toward the outer surface during apoptosis. PS inversion is one of the early markers of apoptosis. It is possible to distinguish apoptotic and necrotic cells by staining with Annexin-V−FITC followed by counterstaining with PI as the former binds PS while PI is permeable only to cells with compromised membrane integrity. The HL-60 cells

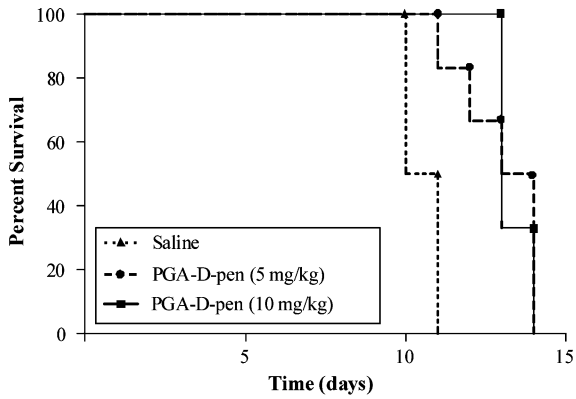

Figure 6. Percent survival curves in CD2F1 mice upon ip administration of PGA−D-pen. Mouse leukemia cells (P388) were implanted ip on day 0. PGA−D-pen conjugate was administered ip on days 1, 5, and 9. Control group (saline) was compared to the groups treated with PGA−D-pen (6 mice/group).

were incubated with three different D-pen equivalent concentrations of PGA−D-pen and analyzed at different time points to determine time and dose dependence. The number of apoptotic cells increased significantly in the PGA−D-pen treated samples (Figure 5) with dose and time. The percent of apoptotic cells upon treatment with 1000 μM PGA−D-pen (D-pen equivalent) for 14 h was 22.5 ± 2.95%.

In Vivo **Evaluation.** The PGA−D-pen conjugate was dosed ip in CD2F1 mice at 4 different dose levels (1, 2, 5, and 10 mg/kg) to determine the acute toxicity. Mice receiving a 10 mg/kg dose showed signs of toxicity (consistently lower BCS scores), but no mortality was observed after 14 days. The mice in this group showed no weight loss, but the mean percent weight gain was less than that of the control group. Therefore, 10 mg/kg was used to evaluate the *in vivo* anticancer efficacy of PGA−D-pen conjugate. The conjugate was also dosed at the 5 mg/kg dose level to determine a dose response in the enhancement of survival.

P388 ip model is a widely used animal model and involves implantation of cells in the ip cavity.[35] The tumor doubling time is 0.4 to 0.5 day, and the survival span is between 9 and 11 days in CD2F1 mice.[36] The median survival upon treatment with 10 mg/kg and 5 mg/kg of PGA−D-pen on days 1, 5, and 9 increased by 28.5% (13.5 days) and 24% (13 days) respectively over control, which was significant ($p < 0.05$) based on log-rank analysis of the survival curves (Figure 6).

Discussion

We and others have previously shown that D-pen is impermeable to cancer cells.[20,37] The impermeability results from a combination of high hydrophilicity (log P −0.39)[38] and the D-isoform being stereochemically less favored for cellular uptake by the amino acid transporters.[37] Polymer bound drugs have been shown to be taken up by the cells via fluid phase endocytosis.[39] Therefore, PGA−D-pen conjugate was synthesized to enhance the intracellular uptake of D-pen. Additionally, conjugation to PGA via disulfide bonds would provide protection to the thiol group of D-pen before it reaches the target cells and ensure intracellular release by endosomal disulfide reduction.[40,41]

D-Pen has been shown to cause cytotoxicity by dose dependent generation of ROS via copper catalyzed one electron oxidation.[3] Cancer cells have an exhausted redox buffer capacity, and further increase in the oxidant load can lead to generation of apoptotic signals that may ultimately lead to cell death. Similar effects were observed with D-pen released intracellularly from the PGA−D-pen conjugate. Thus, it is possible that the major mechanism of cytotoxicity caused by D-pen is apoptosis induction via ROS generation. Leukemia cells were found to be more sensitive to PGA−D-pen treatment with lower IC_{50} values. This is similar to our previously reported *in vitro* experiments[3] except that the cells were not supplemented with copper. To investigate if the ROS generation and cytotoxicity were related to the endogenous copper levels in the different cells, we measured the intracellular copper in HL-60, P388 and MDA-MB-468 cells by ICP-MS (inductively coupled plasma mass spectrometry) (data not shown). There was no direct correlation between the cytotoxicity and the endogenous copper levels suggesting that cytotoxicity of D-pen may be mediated by mechanisms additional to metal catalyzed ROS generation like a P53 dependent apoptosis induction[42] and binding to

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cellular proteins by thiol exchange similar to the endogenous thiols[43] which are all independent of the availability of copper.

The confocal microscopic pictures showed a punctate pattern of fluorescence characteristic of endocytic uptake of macromolecular structures.[39,44] The punctate pattern still exists at 24 h suggesting that endosomal release mechanisms may be needed to release the conjugate or released D-pen into the cytosol. These observations suggest that cytosolic delivery of the conjugate and/or released D-pen may be important. To qualitatively assess the effect of endosomal escape on the cell uptake of the fluorescently labeled PGA−D-pen, uptake studies were performed in the presence of 50 μM chloroquine diphosphate (CD) based on preliminary cytotoxicity studies with CD alone in which this concentration did not result in significant reduction in cell viability. CD is a lysosomotropic agent and has been shown to increase cytosolic delivery[45] by raising the endosomal pH[46] leading to an osmotic burst of the endosome. In our studies (Figure 2D), we found that CD treatment resulted in a diffused pattern of fluorescence of the fluorescently labeled PGA−D-pen in HL-60 cells. Moreover, cotreatment of HL-60 cells (4×10^4) with PGA−D-pen conjugate and 50 μM CD resulted in 20% enhancement in cytotoxicity of the conjugate (at IC_{50} value) when normalized to CD treatment over 48 h suggesting that endosomal release may be important in the enhancement of the efficacy of the conjugate. We are currently investigating cellular uptake and transport mechanisms of the conjugate including possible strategies to avoid endosomal accumulation. Future efforts are focused on incorporation of an endosomal release mechanism that would result in the cytosolic delivery and more efficient intracellular reduction of the conjugate.

Based on the results of studies above, it was concluded that PGA−D-pen was able to successfully deliver D-pen to cancer cells. Although high doses of the conjugate (D-pen equivalent) are required to achieve beneficial effect if used alone, it has been reported that tumorigenic cells are 30- to 100-fold more sensitive to treatment with D-pen compared to normal cells.[42,47] This in-built selectivity makes D-pen a potential agent for development as an anticancer drug. However, it may be essential to combine it with a standard chemotherapy regimen when used in the clinic. For example, NOV-002, a new drug formulation under clinical trials, is the disodium salt of glutathione disulfide in complex with cisplatin in a ratio of 1000:1. It has been proposed that NOV-002 acts mainly by disturbing the cellular redox balance and results in increased efficacy when used in combination with cisplatin and other chemotherapeutics.[48,49] It is important to note that the active component of NOV-002, glutathione disulfide, is not cytotoxic even at very high doses when used alone, and like D-pen, glutathione disulfide is impermeable to the cell membrane.[43] This is relevant as we have found that treatment with D-pen in the presence of copper results in a decrease in cellular thiols and has the potential to perturb cellular redox balance while generating cytotoxic ROS.[3] Therefore, in an attempt to further enhance the efficacy of PGA−D-pen, we are currently developing dual drug conjugates where an anticancer anthracycline derivative will be conjugated to the PGA−D-pen conjugate via acid-cleavable hydrazone linkages. It has been reported that iron complexes of anthracyclines especially doxorubicin catalyze oxygen consumption and ROS generation by thiols.[50] Codelivery of an anthracycline with D-pen bound to a single polymer may synergistically enhance the anticancer efficacy, tolerability and an overall dose reduction of the chemotherapeutic.

Conclusions

A polymeric conjugate of D-pen with PGA was synthesized to achieve enhanced intracellular accumulation and anticancer efficacy. D-Pen was linked to the polymer by disulfide bonds using a heterobifunctional linker. The conjugate increased cell uptake of polymer bound D-pen as observed by confocal microscopy and HPLC. The ROS generation by PGA−D-pen upon cellular uptake results in apoptosis mediated cytotoxicity in P388 murine leukemia cells. PGA−D-pen significantly enhanced the survival of CD2F1 mice over control animals. Future efforts are focused toward investigating the intracellular release of D-pen from the conjugate and improving the anticancer efficacy of PGA−D-pen by synthesizing dual drug conjugates.

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