FUNCTIONALIZED POLYMERS FOR GENE THERAPY: DISCOVERY, OPTIMIZATION AND CLINICAL DEVELOPMENT

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Introduction

Non-viral delivery of therapeutic nucleic acids has become a viable alternative to viral delivery in the field of gene therapy. Cationic lipids and polymers have emerged as the most promising carriers, but due to the complexity of *in vivo* requirements, they must possess multiple functional elements. PPC was designed and optimized to contain a cationic polymer, PEI, derivatized by the covalent attachment of cholesterol and polyethylene glycol (PEG).[1] PPC was used to deliver interleukin-12, a potent immunostimulatory cytokine, gene encoded in a bacterial plasmid to treat recurrent epithelial ovarian cancer. After successful pre-clinical development and demonstration of efficacy in mouse models, IL-12/PPC syntheses were scaled up using GMP methods. A clinical trial was initiated using a combination treatment of IL-12/PPC and carboplatin/docetaxel and has currently moved into phase IA of the trial.

Experimental

Synthesis and analysis of physical/chemical properties of PPC. PPC was synthesized by combining branched polyethylenimine (BPEI, MW 1800) (Polysciences), with cholesteryl chloroformate, and activated methoxypolyethyleneglycol (mPEG-SPA, Methoxypolyethylene-glycol-propionic acid N-hydroxysuccinimidyl ester, mPEG550, ester MW 719, (Nektar Therapeutics). To a stirred solution of BPEI (20 g, 11.1 mmol) in 200 mL of dry $CHCl_3$, a solution containing cholesteryl chloroformate (4g, 9 mmol) and mPEG550-SPA (18.7 g, 26 mmol) in 200 mL of dry $CHCl_3$ was added dropwise over 20–30 min. After 3–4 h, the mixture was concentrated under vacuum. The residue was dissolved in 320 mL of 1 M aq. HCl. This solution of PPC hydrochloride was again concentrated under vacuum to a viscous liquid. PPC hydrochloride was isolated by pouring the concentrated solution into an excess of acetone. The precipitated product was repeatedly washed with acetone. The hygroscopic PPC hydrochloride was dried under vacuum and stored under argon at -20°C until use.

NMR analysis was performed in D_2O at 500 MHz on a Varian-500 spectrometer. The molar ratios of PEI to the functional groups CHOL and PEG were calculated from the NMR data. Polymer DNA complexes were generated by first preparing PPC and DNA in 10% lactose. The DNA was then added dropwise to the stirred PPC solution and incubated for 15 min at RT to form the complexes. Determinations of the complex particle size and ζ-potential were performed by dynamic light scattering using a 90 Plus/BI-MAS Brookhaven particle size analyzer. Specifically, 50 µl of formulation was added to 950 µl of water in polystyrene cuvets for analysis.

DNase protection assay. Free DNA or DNA/PPC complexes were incubated with DNase I (5 IU) for 1, 15, 30 and 60 min at 37°C. An equal volume of termination buffer was added to stop DNase activity. To ~150 ng of the DNase treated DNA/PPC complex was added dextran sulfate (50 µg, MW 10K, 5 mg/ml solution) to separate the DNA from PPC. The mixture was separated in a 0.8% agarose gel. Similar treatment of free DNA was performed as a control.

In vitro analysis of transfection activity. Transfection activity of PPC at various nitrogen to phosphate (N/P) ratios were examined in COS-1 cells. The cells were plated in 12 well plates at 150,000 cells/well in 10% FBS supplemented DMEM medium and grown to ~80% confluency. A total of 4 µg of plasmid encoding luciferase (complexed with various amount of PPC dependent upon the N/P ratio) was added to the cells from a DNA solution of 0.5 mg/mL. An additional amount of FBS-free DMEM was added to bring the total added volume to 500 µl and the cells incubated for 6 h at 37°C. The transfection medium was aspirated and replaced with 1 mL of 10% FBS-supplemented DMEM. The cells were incubated for an additional 48 h and cell lysates were prepared to quantify gene expression. Luciferase activity was determined using the Promega luciferase assay system with a micro plate luminometer (Berthold Detection Systems USA). Total protein concentration in the cell lysates was determined using a BCA assay (Pierce). For mIL-12 gene expression analysis the transfection protocol was the same. Following transfection supernatants were removed and analyzed directly for mIL-12 protein by ELISA (R&D Systems). The luciferase and pmIL-12 plasmids were kindly provided by Dr Sung Wan Kim (University of Utah) and have been previously described.[2,3]

Tumor generation and treatment. The 4T-1 cell lines were established from a spontaneous, moderately differentiated adenocarcinoma growing in Balb/C mice and kindly provided by Dr. Sung Wan Kim. The 4T-1 cells were grown in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate with 10% fetal bovine serum.. For the combination therapy studies involving 4T-1 cells, Balb/C mice were injected with $1x10^6$ cells in 100 µl volume into the left flank only (n =5 per group). One week after implant (when tumors reached ~60 mm^3) chemotherapy treatments were initiated using an investigational chemotherapy reagent called Genexol-PM.[4] Genexol-PM is paclitaxel that has been formulated with a biocompatible polymer for high drug solubility and has been shown to have robust anti-tumor activity in animal models.[5] The pmIL-12/PPC administration began 1 day thereafter. The entire treatment regimen consisted of Genexol-PM treatment (16 mg/kg in 67 µl injected i.v.) on days 7 and 21 after tumor implant and plasmid administrations on days 8, 15 and 22. For ID8 tumor studies, female C57B6/black mice were injected with 5×10^6 ID8(+vegf) cells intra-peritoneally in 500 µl volume. Plasmid treatment began in all animals when the mouse weight (indirect indication of tumor burden) reached approximately 20 grams (~21 days after injection of ID8 cells). The treatment groups ranged from 0- 250 µg plasmid administered. The weight of the animals was determined twice per week.

Results and Discussion

PPC Characterization. PPC was synthesized as a light yellow granular powder that is highly soluble in water. Initially, the reaction was optimized to obtain less than 1 g of material but later scaled up to 50 g lots. The synthesis yield varies between 70% and 90%. Different molar ratios of PEG to PEI (0.6:1 to 18:1) in PPC were targeted by varying the molar concentration of PEG in the reaction mixture. Only the ionic salts (PPC hydrochloride) should be precipitated, while the unreacted mPEG-SPA and cholesteryl chloroformate should remain in solution during acetone washing. Therefore, using the assumption that all of the uncoupled material had been removed, the chemical composition and molar ratio of different components in the PPC molecule were verified by NMR (**Figure 1**). To establish the PEG/PEI/CHOL ratios of PPC, the integration per single proton for each of these moieties is taken for the PPC molar compositional ratio, and normalized to PEI (=1) (**Figure 1**).

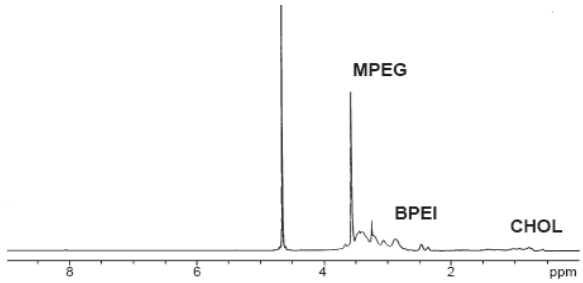

Figure 1. ^{1}H NMR spectrum of PPC.

Effect of PEG/PEI/CHOL ratio and N/P ratio on activity. Polymer composition was initially optimized by evaluating the effect of adding increasing amounts of PEG to the PPC polymer. Results indicate that the addition of (on average) two PEG molecules to every one PEI and one CHOL produced the highest luciferase expression levels following intratumoral injection into 4T-1 murine mammary tumors (**Figure 2**). The expression levels achieved were approximately 10-fold higher than when using polymer containing only PEI–CHOL with no addition of PEG. Addition of PEG beyond the 2:1:1 ratio led to decreased luciferase levels. Analysis of the ζ-potential of the formulations used in these studies indicated a trend towards lower values as the amount of PEG increased and is consistent with a charge masking effect of PEG. Parallel *in vitro*

experiments were performed that were directed at optimizing cell transfection based on the N/P ratios. In COS-1 cells (using PPC 2:1:1) luciferase expression was highest at N/P ratios above 10:1 at which the plasmid DNA was fully condensed by the polymer as indicated by particle size (<100 nm) and ζ-potential (22 mV) and no detectable free DNA by agarose gel electrophoresis.

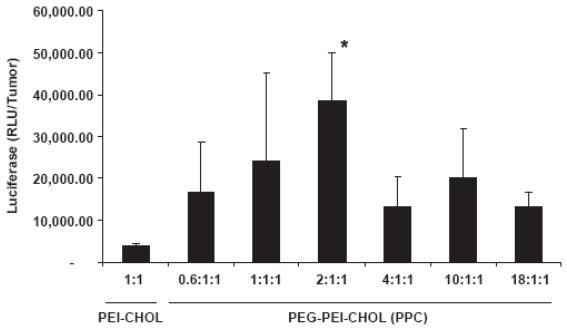

Figure 2. Intratumoral luciferase activity 1 day following intratumoral injections of various polymer/DNA complexes. Mice (3 per group) were implanted with sc tumors into left and right flanks (a total of six tumors per group). Each tumor was then injected with 30 µl of formulated plasmid (0.2 µg/µg DNA at 11:1 N/P ratio). Values are expressed as mean+S.D.

A small (9%) but significant increase in expression levels were observed between 11:1 and 20:1 ratios, however at the latter ratio there was an apparent increase in cytotoxicity as indirectly determined by a decrease (~12%) in the total protein measurements of the cell lysates. It was thus determined that at an N/P ratio of 11:1 the expression was highest with minimal cytotoxicity. When this polymer/DNA complex (11:1) was challenged with DNase, it was seen that the DNA is fully protected for >60 min at 37°C, and no DNA degradation was detected by agarose gel electrophoresis following decomplexation of the polymer and DNA using dextran sulfate. In contrast, unprotected DNA treated in a similar manner was fully degraded following a 1 min incubation with DNase.

Anti-tumor activity of mIL-12/PPC monotherapy and combination therapy. We evaluated the use of mIL-12/PPC at an N/P ratio of 11:1 as in a therapeutic application. *In vitro* it was seen that high levels of mIL-12 are produced in the cell medium following COS-1 cell transfection and are dependent on the amount of DNA used (data not shown). The ability of mIL-12/PPC to inhibit tumor growth was first tested in mice against an established subcutaneous 4T-1 (mammary carcinoma) tumor model. Administrations (3 weekly injections) alone and in combination with an investigational formulation of paclitaxel (Genexol-PM; 2 treatments, every other week) were tested. Genexol- PM treatment alone resulted in significant tumor inhibition compared to untreated controls (**Figure 3**). Greater inhibition was seen when the treatments were combined where an additive effect was seen resulting in nearly complete tumor inhibition.

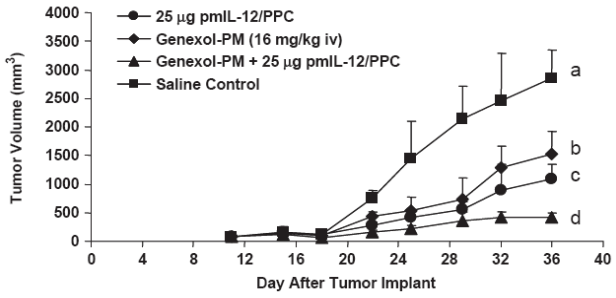

Figure 3. Subcutaneous 4T-1 tumor volumes in female Balb/C mice following treatment. Values are expressed as mean+S.D.with an n =5 for each group.

The same formulation was then used to define a mIL-12/PPC dose response in ID8 tumor bearing mice (*in vivo* ovarian cancer model). mIL-12/PPC complex was administered weekly for four weeks and efficacy was determined by examining both animal weight gain and survival. The effect that mIL-12 treatment had on body mass is shown in **Figure 4**. The increase in body mass over the course of the study is primarily due to the accumulation of ascites fluid in the tumor bearing animals. There is a significant effect of treatment on weight gain as the weight of the animals of all but the lowest treatment group (10 µg). Survival of the animals was monitored and is also represented in **Figure 4**. Median survival correlated with weight gain, with the maximum effect observed at the 50 µg dose and above.

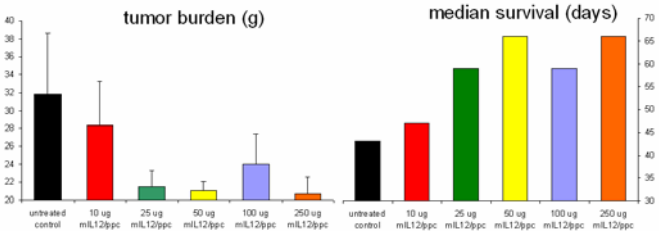

Figure 4. Dose-response of mIL12/PPC monotherapy in ID8-tumor bearing female C57B6/black mice as a function of tumor burden (as determined by animal weight) (left) and median survival (right).

GMP Manufacturing and Scale-up. In order to generated sufficient mIL12/PPC material to transition to a clinical trial, initial syntheses were performed in-house on a 50 g scale. Subsequent commercial PPC synthesis was scaled up to 100 g lots under GMP conditions. Scale up was performed by verifying: the ratio of components (PEI, mPEG, cholesterol) by NMR and FT-IR, water content, elemental analysis, residual solvent, endotoxin, and bioburden. Likewise under commercial GMP conditions, mIL12 plasmid and the mIL12/PPC complexes were scaled up to 5 g lots. The mIL-12/PPC complexes were verified across lots by measuring both particle size and *in vitro* transfection activity.

Summary of Clinical Pharmacology. Human IL-12/PPC (EGEN-001) is currently in clinical development for the treatment of recurrent ovarian cancer. A phase I study of human IL-12/PPC safety and tolerability following intraperitonea (i.p.) administration in women with chemotherapy resistant recurrent advanced ovarian cancer has been completed. A phase IA study of the safety and tolerability of i.p. human IL-12/PPC in combination with carboplatin/docetaxel in women with platinum-sensitive recurrent ovarian cancer is in progress. To date, human IL-12/PPC administration alone or in combination with chemotherapy is well tolerated in ovarian cancer patients. The most commonly experienced adverse events were mild fever and abdominal pain observed during first several hours after the treatment and resolved within 24 hr. The delivery of IL-12/PPC produced local increase in anticancer cytokines. A dose dependent increase in survival benefit was also observed with IL-12/PPC treatment.

Conclusions. The preclinical pharmacology studies have demonstrated significant antitumor activity following local administration of a formulation of murine IL-12 with a new multi-functional polymer, PPC. mIL-12/PPC is effective in several mouse models of cancers including ovary, breast, brain, colon, pancreas and head and neck. mIL-12/PPC treatment inhibited tumor growth and prolonged survival in tumor bearing mice. Local tissue reactions are consistent with the pharmacological action of IL-12 in stimulating the proinflammatory cytokine cascade.

References.

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