

Biodistribution and Clearance Following Intraperitoneal Injection of Murine Interleukin-12 Plasmid Formulated with a Novel Polymeric Delivery System

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ABSTRACT [286]

Intraperitoneal delivery of an Interleukin-12 (IL-12) plasmid formulated with a novel polymeric delivery system (PPC) into nanocomplexes has shown efficacy in treating a variety of disseminated peritoneal malignancies in mice. Pre-clinical GLP studies were conducted to evaluate the biodistribution of plasmid following intraperitoneal administration of increasing doses of PPC formulated murine IL-12 plasmid into CD-1 mice. In the study a total of 54 male mice and 54 female mice were evaluated. Animals were divided equally into control group, low dose (10 µg; ~1.5 mg/ml) and high dose (250 µg; ~38 mg/ml) groups. The doses selected for this study were based upon preliminary toxicology studies and represent significant multiples of the starting dose currently being used in Phase I clinical testing. Specifically the low and high doses are approximately 2.5 and 60 times the starting dose in humans based on a 25 g mouse and a 70 kg human. Animal tissues were harvested at 3, 30 and 88 days after treatment and analyzed using a qPCR assay that was determined to be sensitive to ~10 copies of plasmid/g DNA and quantifiable at >100 copies/g DNA. A total of 11 samples were collected from each animal and included: injection site, blood, gonads, liver, heart, kidneys, lungs, mesenteric lymph nodes, spleen, left femur and brain. The results indicated that following IP dosing, plasmid was detectable in all examined tissues with the highest levels located in the organs of the peritoneal cavity and at the site of injection. Considerably lower plasmid levels were found in the blood, heart, femur (marrow) and brain. Distribution did not appear to be affected by dose with proportional levels found in all tissues at the different doses. An approximate 95% clearance occurred within 1 month with 99% clearance by three months in almost all tissues, however slightly higher levels remained at the sight of injection after 88 days. In summary these data indicate that plasmid formulated with PPC and injected IP will lead to both local and systemic uptake of plasmid. However, the plasmid is readily cleared from all tissues over time.

INTRODUCTION

Immunotherapy strategies that involve cancer treatment by stimulation of the body's immune system to fight against cancer have gained acceptance during the last decade. Interleukin-12 (IL-12) has proven to be one of the most active cytokines for inducing potent anti-cancer immunity (Robertson and Ritz, 1996). Interleukin-12 is associated with immunomodulatory properties including T-lymphocytes and natural killer (NK) cell proliferation and cytotoxic activation and secretion of interferon-gamma (IFN-γ) subsequently leading to tumor inhibition. Interleukin-12 also potentiates T helper cell differentiation and development of cytotoxic CD8+ T cell responses against viruses and tumor cells. Despite initial aggressive testing in the clinic, use of recombinant IL-12 therapy has not advanced to an approved therapy due primarily to systemic toxicity concerns, which has led researchers to explore alternative means of IL-12 delivery (Imboden et al., 2003; Kang et al., 2001).

We have developed a non-viral gene delivery approach composed of an IL-12 gene and a novel polymeric delivery system. Results from *in vivo* studies have indicated that this system is useful for treating a variety of solid tumors and peritoneal malignancies.

The purpose of this study was to determine plasmid biodistribution of pml-12 (a murine IL-12 DNA plasmid) formulated with the novel polymeric gene carrier PPC (PEI-PEI-CHOL) at 3, 30, and 88 days after a single intraperitoneal (IP) injection to male and female CD-1 mice. Three groups of CD-1 mice (18 animals/group) were administered 0.5 mL of either 10% lactose solution or 0.5 mL of a 20 or 500 µg/mL formulation of pml-12 (PPC) by IP injection. Six animals/group were sacrificed on study day 3 or 30, and all remaining animals were sacrificed on study day 88. The results of this study have been used to support initiation of a Phase I clinical trial evaluating IP delivery of human IL-12/PPC in women with recurrent ovarian cancer.

MATERIALS

The novel lipopolymer PPC (FIGURE 1A) is composed of a low molecular weight branched polyethyleneimine (PEI) covalently linked with the functional groups methoxyethylpolyethylene glycol (PEG) and cholesterol (CHOL). Synthesis details can be found in (Fewell et al., 2005).

The murine IL-12 plasmid (FIGURE 1B) contains both the p35 and p40 genes each under control of a separate CMV promoter/enhancer. Each gene is followed by the 3'UTR late polyadenylation signal sequence.

Plasmid and PPC were formulated at a 1:1 nitrogen to phosphate ratio and lyophilized. The final solution was reconstituted at 0.5 mg/mL with water for injection prior to use.

The design of the study is outlined in FIGURE 2 and TABLE 1. The housing of the animals, the injection of the test articles, and the harvesting of the tissues was performed by Gene Logic, Inc. (Gaithersburg, MD) following an approved IACUC protocol and conforming to GLP guidelines. The biodistribution qPCR assay was performed by Althea Technologies, Inc. (San Diego, CA) utilizing aseptic techniques and conforming to GLP guidelines.

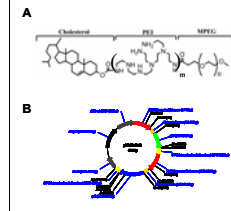


FIGURE 1
The novel lipopolymer PPC (A) and the murine IL-12 plasmid (B).

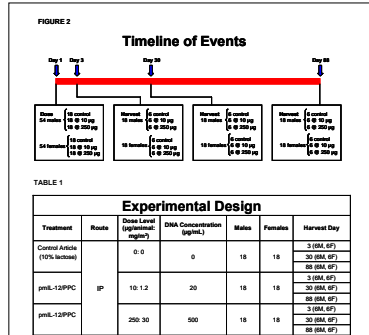


FIGURE 2 and TABLE 1
Schematic representations of the timeline of events and the experimental design. 108 mice were given a single IP injection of either the control article (10% lactose) or one of two doses of the test article (0.5 mL of a 20 or 500 µg/mL formulation of pml-12/PPC). 36 mice each day were harvested for analysis on study days 3, 30 and 88.

METHODS

A quantitative polymerase chain reaction (qPCR) assay was developed and qualified to detect a specific target sequence of mIL-12 plasmid DNA in mouse tissue. The assay was developed to provide maximum sensitivity and specificity for detecting the target gene sequence in a background of 1 µg mouse genomic DNA (gDNA). The assay detects a 148 nucleotide sequence of the pml-12 DNA. The 5' PCR primer is located in the region that spans the chimeric intron and the p35 gene insert (AATCTATGTGTCAATACAGCTACTCT). The 3' PCR primer is located within the p35 gene insert region (CTGAAGACCACAGATGACATG). The fluorescently labeled probe is located within the detected sequence.

A standard curve was prepared using pml-12 DNA, diluted in a background of mouse liver gDNA. The standards used were: 1×10^6 copies pml-12 plasmid DNA per microgram of mouse liver gDNA (copies/µg), 1×10^4 copies/µg, 1×10^2 copies/µg, 1×10^0 copies/µg, 10 copies/µg, 0 copies/µg. The specificity of the assay was established using these negative controls: 1 µg negative (0 copies) mouse liver gDNA and a no template control. Reactions were performed in triplicate and each plate contained one set of standards, one negative control and one no template control. The cycling conditions for the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) are as follows: hold at 50°C for 2 minutes, hold at 95°C for 10 minutes, and 45 cycles of 95°C for 30 seconds, 50°C for 1 minute.

Results were reported as the number of copies of the target per µg DNA. The range of quantification of the assay is from 100 to 1×10^4 copies/µg DNA. Samples that tested below the limit of detection (10 copies/µg DNA) were identified as "LLD" (less than the limit of detection). Samples that tested between 10 and 100 copies were below the limit of quantification of the assay and were identified as "NQ" (not quantifiable). Results that exceeded the upper limit of the dynamic range of the assay were identified as ">10,000 copies/µg DNA".

RESULTS

- Plasmid DNA was detected in all examined tissues following intraperitoneal administration of the plasmid.
- The highest levels of plasmid DNA were located in organs of the peritoneal cavity: gonads, liver, mesenteric lymph nodes, and spleen.
- Less plasmid DNA was found in blood, heart, femur (marrow), and brain suggesting limited systemic exposure following intraperitoneal injection.
- No detectable plasmid DNA was found in blood and tissues following intraperitoneal administration of the control article.
- Clearance of 95-98% of the plasmid occurred within approximately one month following injection. Further clearance of the plasmid DNA occurred over the subsequent two-month interval during which more than 99% was cleared from the tissues, indicating that the plasmid underwent a rapid and extensive clearance following intraperitoneal administration.
- The distribution of plasmid DNA in tissues collected at all intervals was identical in that the highest levels were found in gonads, liver, mesenteric lymph nodes, and spleen. Therefore, the distribution was unaffected by dose.

Biodistribution of plasmid DNA

Males	Tissue	Females
-	Brain	-
-	Blood	-
+	Heart	+
+	Left Femur	+
++	Kidneys	++
++	Gonads	++
++	Lungs	++
+++	Liver	+++
++++	Injection Site	++++
++++	Mesenteric Lymph Nodes	++++
++++	Spleen	++++

TABLE 2
Quantitative estimate of the biodistribution of pml-12 plasmid DNA in various tissues in the 10 µg dose group from the day 30 harvest.

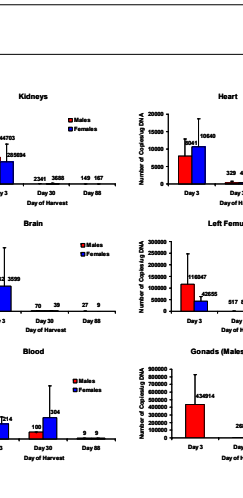


FIGURE 3
Expression levels of pml-12 plasmid DNA in various tissues in the 10 µg dose group after a single IP plasmid administration. The levels of pml-12 DNA were determined by qPCR analysis with values expressed as average number of copies of plasmid DNA per µg of mouse genomic DNA. For day 3, n = 5 mice per group; day 30, n = 6; day 88, n = 5. To determine expression levels, a result of LLD (>10 copies/µg) was assigned a value of 9. A result of NQ (10-100 copies/µg) was assigned a value of 100.

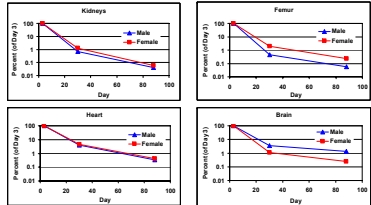


FIGURE 4
Estimated clearance of pml-12 plasmid DNA from various tissues over 85 days following a single IP administration in male and female CD-1 mice. From the qPCR assay, average values for each tissue were obtained of number of copies of plasmid DNA per µg mouse genomic DNA. Percent values were calculated by comparing the averages of day 3, day 30 and day 85 for each tissue.

Percent Clearance over 85 Days

	Heart	Left Femur	Kidneys	Brain
Males	99.7%	99.9%	99.9%	98.6%
Females	99.6%	99.8%	99.9%	98.7%

TABLE 3
Percent clearance of pml-12 plasmid DNA from various tissues over 85 days following a single IP administration in male and female mice. The percent values were obtained by comparing day 3 to day 88 averages.

CONCLUSIONS/SUMMARY

- PPC, a novel polymer, was optimized to produce stable nanocomplexes with DNA leading to local and systemic uptake of plasmid.
- The administration of a plasmid encoding for murine IL-12 formulated with the PPC delivery system has been shown to be well tolerated in mice following IP delivery.
- Intraperitoneal injection of pml-12 formulated with PPC produces high levels of mIL-12 that persist for several days.
- A plasmid encoding for human IL-12 and formulated with PPC (EGEN-001) is currently being evaluated in an open labeled Phase I clinical trial in women with recurrent ovarian cancer that are not responsive to chemotherapy. In this trial escalating doses of plasmid are being administered IP on a weekly basis for up to eight weeks. Recruitment for this multi-center trial is ongoing, with preliminary results to be presented Sunday June 4th.

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