## D. Research Design and Methods

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We propose a novel method to specifically deliver gemcitabine polymers to pancreatic cancer cells, increasing its efficacy and decreasing toxicity to normal cells. This approach takes advantage of (1) the observation that RNA aptamers that bind epidermal growth factor receptor (EGFR) are internalized by cells that express EGFR (Preliminary Data); (2) the fact that gemcitabine is a nucleoside and is therefore amenable to polymerization and to direct conjugation to the EGFR aptamer; and (3) can thereby facilitate targeted delivery. *We hypothesize that aptamer-mediated delivery of gemcitabine polymers will help to bypass some of the primary mechanisms of gemcitabine resistance by delivering high concentrations of gemcitabine monophosphate directly to the cells that need it.*

This project was initiated as a collaborative effort between the PI (Dr. [redacted], a surgical oncologist whose clinical practice and translational research efforts are focused on pancreatic cancer, and Dr. [redacted] at University of [redacted] a biochemist whose laboratory developed the EGFR aptamer and who has extensive experience with RNA therapeutics and nucleic acid polymerases. There are multiple conceivable ways to polymerize and attach gemcitabine to an RNA aptamer, and we plan to explore several approaches in parallel, since different approaches may result in products with different biological properties, including extracellular stability, cellular uptake, intracellular stability, and cytotoxicity. Dr. Barbara Ramsay Shaw, a chemist at Duke, has been included as a collaborator to take advantage of her expertise in nucleotide chemistry, particularly the synthesis and manipulation of boranophosphate nucleotides.

### E. 1. Specific aim #1: Generate EGFR aptamer:gemcitabine polymer constructs.

*E. 1. a. Rationale*

While we can potentially rely upon covalent conjugation to link gemcitabine to aptamers, this approach would be costly (one molecule of gemcitabine per aptamer) and unlikely to be more effective than gemcitabine alone. Since gemcitabine is a nucleoside, we propose to generate gemcitabine monophosphate (dFdCMP) polymers that can be attached to the end of an aptamer. Each step can potentially be performed enzymatically

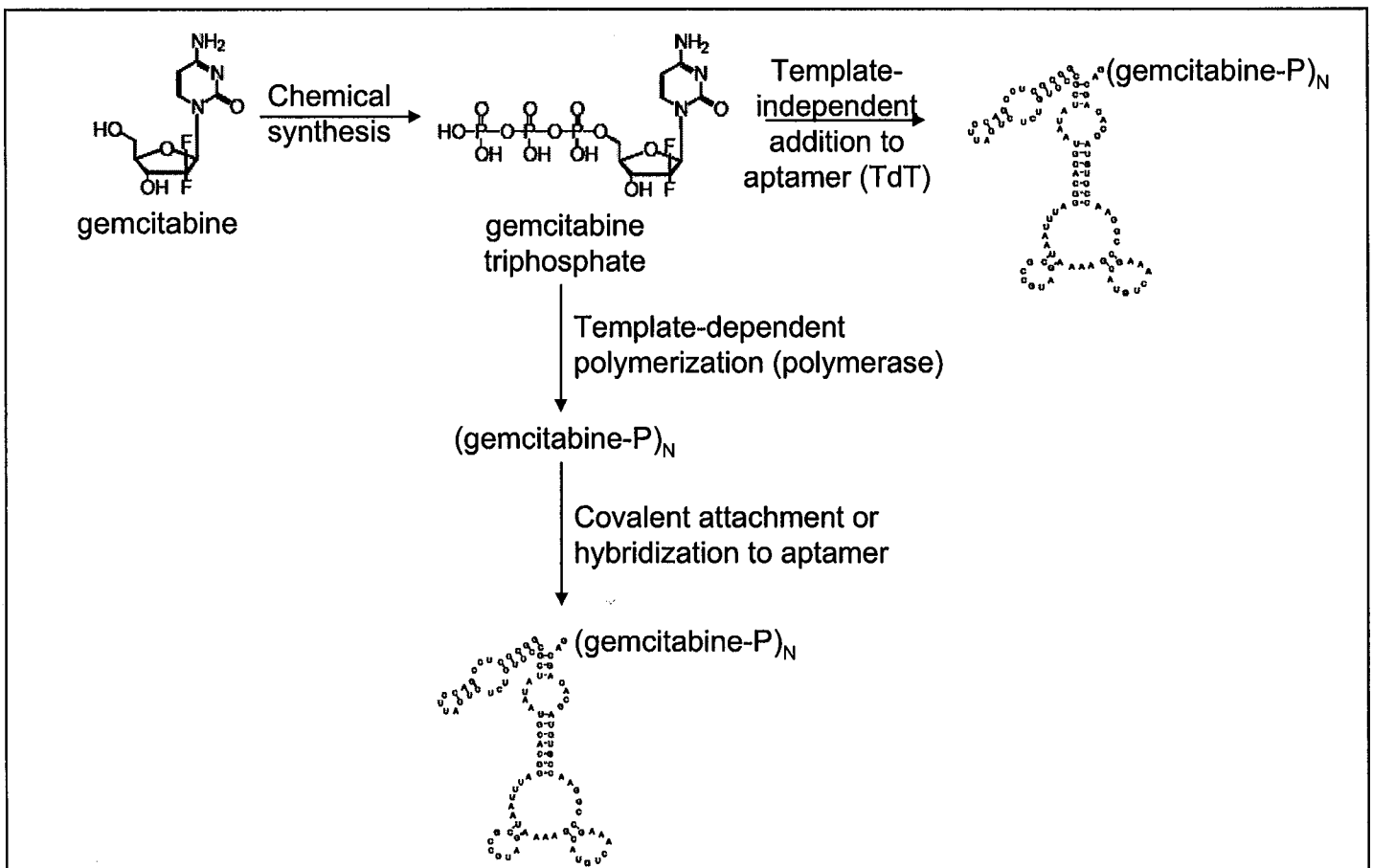


Figure 5. Schematic of approach to synthesis of aptamer:gemcitabine construct. TdT=terminal deoxynucleotidyl transferase. $(Gemcitabine\text{-}P)_N$=gemcitabine monophosphate polymer.

or chemically, and the ultimate method of production will be dictated both by efficiency and efficacy, which will be dependent on the intracellular location and rate of hydrolysis to monomers. Figure 5 depicts a simple schematic of several envisioned approaches.

*E. 1. b. Generation of phosphodiester gemcitabine polymer constructs involves synthesis of the triphosphate, followed by enzymatic polymerization. (Fig. 5)*

Gemcitabine is a pyrimidine *nucleoside* analog (2',2'-difluoro, 2' deoxycytidine or dFdC) and is commercially available [redacted]. By analogy with enzymatic conversions of nucleosides *in vivo*, the first step in the cellular process is the multistep, enzymatic conversion of dFdC to dFdCTP. *Specifically*, dFdC is converted to dFdCMP by dCK, then to the diphosphate (dFdCDP) and finally to the dFdCTP by other cytoplasmic kinases, where it is incorporated into DNA. While this process can be reproduced *in vitro*, nucleosides can also be converted more readily to triphosphates by a variety of chemical means. We propose the following methodology:

(1) Chemical synthesis of the precursor triphosphate analog of gemcitabine. For synthesis of small molecules like nucleotides, chemical synthesis of the gemcitabine triphosphate will be more efficient than enzymatic synthesis. Dr. [redacted] laboratory, in collaboration with [redacted] at University of [redacted], has utilized a traditional two-step [redacted]) reaction to generate dFdCTP from dFdC via dFdCMP. Dr. [redacted] laboratory is utilizing a single-step modified [redacted] method to generate dFdCTP directly from dFdC(He et al., 1998). Nucleotides are purified by anion exchange chromatography; then each fraction is collected and analyzed by liquid chromatography/mass spectrometry (LC/MS with ion trap detector). The nucleotides are lyophilized and then further purified by ion-pairing reverse phase high pressure liquid chromatography.

(2) Enzymatic generation of phosphodiester-linked gemcitabine polymer constructs (see Fig. 5) . dFdCTP is known to be incorporated by eukaryotic polymerases into DNA, and it is therefore possible that dFdCTP will also be incorporated by a variety of other enzymes known to incorporate nucleotides.

(i) Therefore, Dr. [redacted] laboratory will initially attempt to add dFdCTP directly to the 3' end of the aptamer in a template-independent manner using terminal deoxynucleotidyl transferase (TdT). Addition of dFdCTP by TdT would result in a mixture of chain lengths that could be regulated to some extent by controlling the reaction time and the ratio of reagents.

(ii) Alternatively, mutant prokaryotic RNA polymerases (T7) are routinely used for the *in vitro* synthesis of RNA containing non-canonical 2'-modified pyrimidine nucleotides [redacted] 1999). It is likely that dFdCTP could be incorporated by transcription using these or other even more permissive [redacted] 2004) mutant polymerases. This strategy would require a DNA template and could therefore be used to produce very specific chain lengths. As a cytidine analog, we assume that dFdCTP will base pair with guanine nucleotides (G). [redacted] laboratory will initially use this T7 approach, with natural NTPs and with boranophosphate NTPs, which are good substrates for DNA and RNA template-directed polymerases. The TdT approach will also be tried with the borano-modified dFdCTP substrate synthesized in their lab.

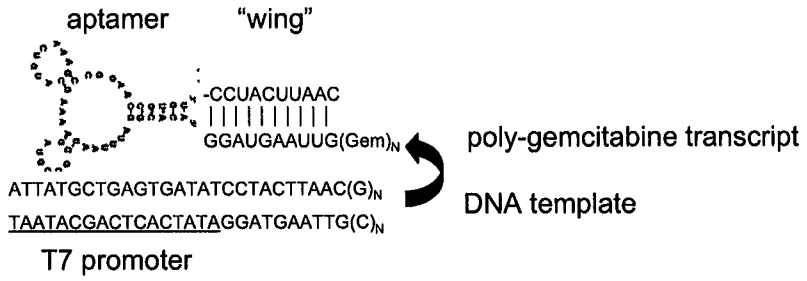


Figure 6. Gemcitabine polymer may be transcribed from DNA template then annealed to aptamer using complementary "wing" sequence.

(iii) Dr. [redacted] laboratory has designed a series of constructs (Figure 6) in which a G-rich DNA template is preceded by a sequence encoding a T7 consensus promoter sequence (TAATACGACTCACTATA...) and a short sequence (10-20 nucleotides) complementary to either the 3' end of the aptamer or a 3' "wing" extension of the aptamer. The resultant transcript would consist of a short sequence that could be used to anneal the polymer to the aptamer followed by the gemcitabine-rich polymer. We have used a similar 24-nucleotide "wing" construct for labeling the full-length EGFR aptamer for flow cytometry without significant decrement in binding affinity. In fact, we believe this "wing" sequence may serve as a spacer that prevents the attachment from folding back and interfering with the aptamer sequence (and hence increases the probability that the aptamer will assume the same 3-dimensional structure and specificity as the "wingless" sequence). Since we do not know what the ideal gemcitabine:aptamer ratio will be, we will initially create constructs with ratios ranging from 10 to 100.

We can subsequently create constructs with even higher ratios if the smaller constructs are confirmed to be internalized by cells.

*Pitfalls and Alternatives*

1. Although chemical polymerization of nucleotides via standard phosphoramidite technology is always an option, there are several limitations to this approach. For one, the current limit of efficient oligonucleotide chemical synthesis is on the order of 100 nucleotides, whereas nucleic acid sequences of greater than 1000 nucleotides can be "synthesized" enzymatically *in vitro* using polymerases. We really do not know what the ideal gemcitabine:aptamer ratio will be but anticipate that a higher ratio will be more effective. By contrast, enzymes have higher fidelity, and—particularly for longer oligonucleotides—the enzymatic product will be cleaner, which is particularly important for early proof-of-concept experiments when we want to know exactly what we are testing. An additional issue for boranophosphate nucleotides, which may assist with delivery or hydrolysis of the gemcitabine polymer, is that chemical polymerization results in stereo-specific Sp isomers, which are avoided with enzymatic polymerization.

2. In order to streamline the transition to *in vivo* studies, we ultimately would like to be able to use an aptamer that is amenable to large-scale chemical synthesis. The current minimized EGFR sequence of 48 nucleotides is amenable to chemical synthesis (and is, in fact, not amenable to enzymatic synthesis by T7 polymerase due to the lack of at least two G's immediately following the promoter). However, the addition of a 10-20 nucleotide "wing" sequence may render chemical synthesis inefficient and cost-prohibitive. Based on the predicted secondary structure of the minimized aptamer, we would expect that directly annealing the gemcitabine polymer to the aptamer would interfere with its structure and therefore function. We will need to empirically determine the minimal length of wing sequence necessary to anneal a long cytidine polymer without interfering with aptamer function. If the current aptamer/wing sequence is too long to be chemically synthesized, further minimization of the aptamer may be necessary for *in vivo* studies. Meanwhile, for *in vitro* proof-of-concept studies, we can likely retrofit the aptamer by making substitutions within the terminal stem of the 48-nucleotide truncate that would place at least two G's to the 5' end and allow transcription by T7 polymerase or use an alternate polymerase.

3. Although the "G-rich" region of the template would ideally be entirely G (so that the transcript would be predominantly gemcitabine), we have encountered some difficulty amplifying and transcribing such sequences due to their tendency to form quaternary structures. Therefore, it may be necessary to interpose T into this region (e.g., $GT_n$, $GGT_N$, $GGGT_N$).

*E. 1. c. Generation of boranophosphate gemcitabine polymer constructs*

We expect that EGFR aptamer:gemcitabine constructs will be internalized by cells via endocytosis, as EGFR has long served as a model for ligand-induced, receptor-mediated endocytosis[redacted] 1990). While the success of this approach relies on the stability of the construct prior to cellular uptake, once the construct is within the cell, stability is no longer desirable. Within the endosome, the drug will be released by the actions of endosomal nucleases and the acid pH (< 6) of the endosome. Importantly, the drug may be released in its monophosphate form, bypassing the rate-limiting step of conversion by dCK. The drug would then be free to diffuse into the cytoplasm and induce cell death. It is this requirement for extracellular stability and intracellular instability that makes boranophosphate polymers potentially preferable to phosphodiester polymers (Figure 7). Boranophospate oligonucleotides have been explored for RNA therapeutics due to their even greater resistance to endonuclease degradation and greater lipophilicity than phosphorothioate oligonucleotides[redacted], 2000). It has also been observed that boranophosphate linkages are more susceptible to cleavage in acid environments (such as the endosome)[redacted] 2000) and thus might release greater amounts of dFdCMP. Boranophosphate nucleotides share with phosphorothioate nucleotides the ability to

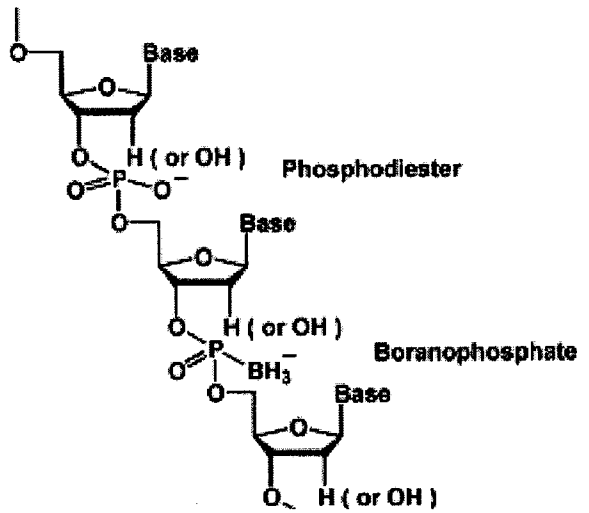


Figure 7. Phosphodiester and boranophosphate nucleotides are iso-electronic and iso-ionic.

mimic normal phosphodiester nucleotides by being readily incorporated into RNA and DNA by polymerases. Furthermore, some mutant viral polymerases have a 50-fold preference for boranophosphate NTPs as substrates relative to natural NTPs. Dr. [redacted] laboratory has expertise in the synthesis of boranophosphate nucleotides([redacted], 1998), which can then be polymerized using the same approaches described above for phosphodiester nucleotides. Boranophospate nucleotides also have a unique property in that boron, when it interacts with neutrons, releases locally destructive alpha particles and lithium nuclei without ionizing radiation([redacted], 2006). Aptamers could deliver boron-containing gemcitabine polymers specifically to cancer cells, where the gemcitabine will be released and incorporated into DNA. This is therefore an ideal delivery agent for boron capture neutron therapy, which could potentially be used to "clean up" cells that are resistant to even high doses of gemcitabine. Although evaluation of this agent for boron capture neutron therapy is not a realistic goal within the two-year timeframe of this project, it is a realistic medium-term goal.

**E. 2. Specific aim #2: Evaluate the effects of EGFR aptamer–gemcitabine constructs on pancreatic cancer cell growth and normal cell toxicity *in vitro*.**

Using standard "double-filter" *in vitro* quantitative binding assays([redacted] 1993), the binding affinities of aptamer:gemcitabine constructs will be compared to the binding affinity of the aptamer alone to ensure that the addition of gemcitabine polymer does not affect binding. Mutant (non-binding) aptamer:gemcitabine constructs will be made in parallel to serve as a control for *in vitro* and *in vivo* assays.

The aptamer:gemcitabine constructs will first be evaluated using *in vitro* proliferation and apoptosis assays. We already possess all 19 human pancreatic cancer cell lines commercially available from ATCC and have performed gemcitabine sensitivity assays (Figure 8) on all 19 lines. We will first utilize a pancreatic cancer cell line that expresses high levels of EGFR and is relatively resistant to gemcitabine (e.g., Panc-1) to screen various aptamer:gemcitabine constructs for activity (in comparison to aptamer alone). Once we have identified one or a few constructs with activity, we will evaluate other pancreatic cancer cell lines, spanning a range of EGFR expression levels, and compare the effects of the constructs on lines that are relatively resistant to gemcitabine to lines that are relatively sensitive to gemcitabine (e.g., BxPC3). Cell lines will be treated with EGFR aptamer:gemcitabine constructs, mutant (non-binding) aptamer:gemcitabine constructs, gemcitabine alone, or EGFR aptamer alone at concentrations ranging from 0 to 50 μM, and the 50% inhibitory concentration (IC50) will be determined using the fluorimetric CellTiter-Blue Cell Viability Assay (Promega). In parallel, apoptosis will be measured using the fluorimetric Sensolyte Rh110 caspase 3/7 assay (Anaspec).

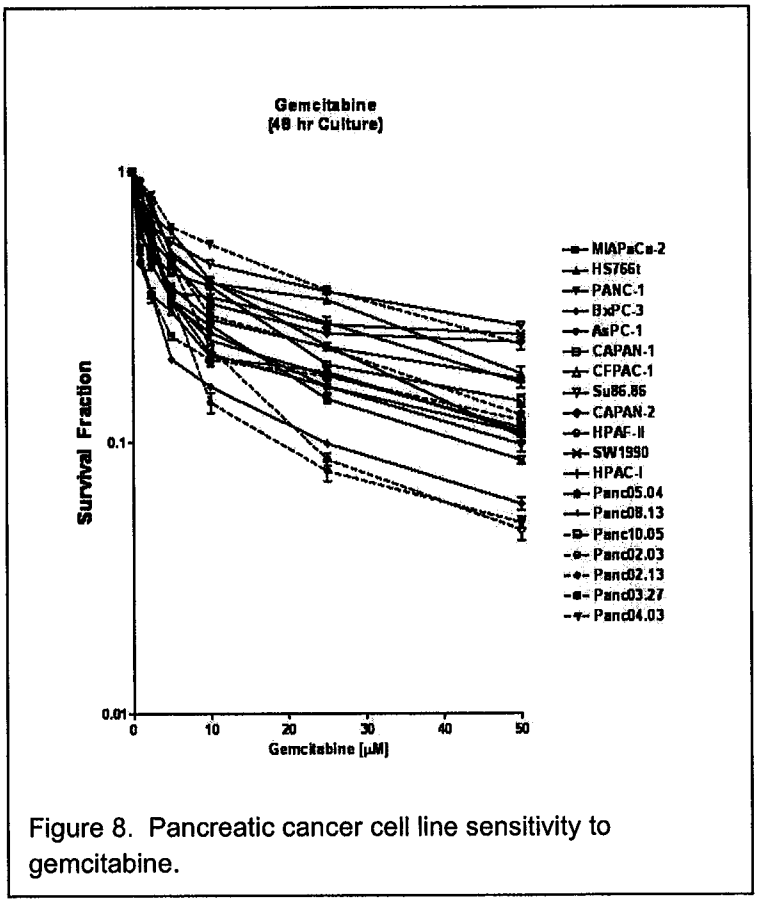


Figure 8. Pancreatic cancer cell line sensitivity to gemcitabine.

Since the most common dose-limiting toxicity of gemcitabine in pancreatic cancer patients is hematologic toxicity([redacted] 1997), we will also measure gemcitabine levels in human peripheral blood mononuclear cells (PBMCs) that have been treated with EGFR aptamer:gemcitabine constructs using anion exchange LC/MS as previously described([redacted] 2006). By comparing gemcitabine levels in PBMCs that have been treated with aptamer:gemcitabine constructs to PBMCs that have been treated with gemcitabine alone, we hope to directly confirm that not only is the aptamer able to increase delivery to EGFR-overexpressing cancer cells but that it reduces uptake in normal cells.

*Pitfalls and Alternatives*

If the gemcitabine monophosphate polymer is not efficiently internalized by cells, it is possibly related to excessive negative charge. Efficiency of internalization may be improved by decreasing the length of the

polymer chain. Another alternative would be to directly polymerize the gemcitabine nucleoside, which upon hydrolysis would still require cellular dCK, but would carry significantly less charge and would likely be internalized more efficiently.

**E. 3. Specific aim #3: Evaluate the effects of EGFR aptamer–gemcitabine constructs on pancreatic cancer tumor growth and normal cell toxicity *in vivo*.**

EGFR aptamer – gemcitabine constructs with promising *in vitro* activity will be evaluated *in vivo*. Although 2'-fluoro-modified RNAs are resistant to nuclease degradation, the circulating half-life is limited by renal clearance and is directly related to size. The addition of polyethylene glycol (PEG) to the 5' end of RNAs of similar size and composition has been shown to significantly decrease renal clearance, with complexation to 20 kDa PEG providing optimal biodistribution [redacted]., 2004). Therefore, prior to transitioning to *in vivo* studies, the EGFR aptamer will ideally be formulated for systemic delivery by complexation with 20 kDa PEG. Since the addition of high molecular weight moieties can have unpredictable effects on aptamer structure, we will need to confirm that the modification does not significantly affect the EGFR aptamer's binding affinity or ability to be internalized.

For our *in vivo* experiments, we will use the human pancreatic cancer cell line Panc-1, which—in addition to overexpressing EGFR and being relatively resistant to gemcitabine—is highly tumorigenic. Xenografts will be established in the flanks of nude mice by injection of $10^6$ cells in 50% Matrigel (BD Biosciences). When the tumors are easily palpable (typically ~ 6-7 mm in diameter), the mice will be randomized to treatment with gemcitabine 100 mg/kg twice a week intraperitoneally for four weeks, EGFR aptamer:gemcitabine construct, mutant aptamer:gemcitabine construct, or vehicle. If EGFR aptamer alone demonstrates growth inhibitory effects *in vitro*, this will also be included as a control in the animal experiments. The twice a week intraperitoneal dosing of gemcitabine is a standard dose that has been used to induce growth delay in pancreatic cancer xenograft models [redacted] 2006). We have delivered aptamers of similar composition by intraperitoneal injection [redacted]., 2008) and will use the same route and schedule for the aptamer treatment groups as for gemcitabine to facilitate comparison. The dose(s) of aptamer: gemcitabine construct will be selected based on *in vitro* cytotoxicity assays but would be expected to be much lower than the molar dose of gemcitabine alone and will need to be, in order for these animal experiments to be feasible. Relative tumor growth inhibition (TGI) at four weeks will be calculated as the average volume of the treated tumors divided by the average volume of the control tumors. Daily weights and behavior of the mice will be monitored, and blood and tissue will be collected at the time of sacrifice for assessment of toxicity (specifically, complete blood cell counts and liver biopsies). Subsequent experiments will include cell lines with lower or no significant overexpression of EGFR to evaluate the specificity of the EGFR aptamer:gemcitabine constructs for EGFR-expressing tumors and dose-response experiments to establish optimal dosing, route, and schedule.

*Pitfalls and Alternatives*

If complexation to PEG significantly affects the aptamer's function or if complexation to PEG is not feasible, there are at least two alternatives. For one, attachment of the gemcitabine polymer to the aptamer may increase its molecular weight sufficiently that complexation to PEG is unnecessary, and we will test the un-PEGylated aptamer *in vivo*. If the gemcitabine polymer is relatively short (i.e., less than 60 nucleotides or 20 kDa) and we are concerned that circulating half-life will be insufficient to accurately assess efficacy, we can increase the frequency of dosing or administer treatment via an implantable osmotic pump (Alzet) that will allow us to maintain constant plasma levels in each treatment group without having to account for the very different pharmacokinetic properties of gemcitabine, aptamer alone, and aptamer: gemcitabine constructs.

**Future Plans and Summary**

Although the ultimate goal of this research is to develop a promising RNA therapeutic approach that can be translated to human patients with pancreatic cancer, the proposed research will potentially identify approaches that can be applied to other nucleoside analogs, other nucleic acid aptamers, and other tumor types. The PI is also a surgical oncologist who treats patients with pancreatic cancer and, as a member of the [redacted], has access to the infrastructure for preclinical development. Although systemic delivery of aptamers in humans is possible, local (i.e., endoscopic ultrasound (EUS)-guided injection into the primary tumor) or regional delivery (i.e., hepatic arterial infusion) in patients with hepatic metastases

are also options that may be less costly for an initial proof-of-concept clinical study. The application of RNA therapeutics to cancer patients is therefore not necessarily a *long*-term goal.