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Research Article

CHK1 siRNA Containing Gemcitabine and Chemically Modified Nucleotides Shows Antitumoral Activity *in Vitro* and *in Vivo*

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Abstract

Gemcitabine was shown to augment anti-cancer activity when combined with small molecule CHK1 inhibitors. We had previously demonstrated that we could incorporate gemcitabine molecules into the backbone of an siRNA targeting CHK1 and studied this drugs effects in pancreatic cancer cells [1]. We had optimized the number of gemcitabines to improve efficacy in the combination where the CHK1 siRNA was unmodified [1]. In this manuscript, we extend these studies by using chemically modified siRNAs using the same siRNA sequence targeting CHK1. We varied the 2'-Fluoro and 2'-O-Methyl modification patterns within the sequence, with gemcitabines in the same locations within the same siRNA sequence. We show that adding gemcitabines into the siRNA can demonstrate improved potency, not only against pancreatic cancer cell models but also against lung cancer, ovarian cancer, triple negative breast cancer and colon cancer.

We formulated the different versions of CHK1 siRNA + gemcitabine into polypeptide nanoparticles with good control over size (~82-88nm) and zeta potential (34-36mV). When delivered intravenously to mice bearing a pancreatic xenograft tumor, the cocktail showed a reduction in tumor burden. The product demonstrated inhibitory effects in vivo, with no effect on the body weights of the animals.

We further examined the ability to improve on the potency of this reagent by inclusion of a second siRNA (targeting WEE1) and demonstrate that, in vitro, the pancreatic cancer cell line, MiaPaca2, is particularly sensitive to a combo of CHK1 siRNA plus gemcitabine together with a second siRNA against WEE1.

Keywords: SiRNA, CHK1, Gemcitabine, Nanoparticle Delivery, Xenograft Tumor, Pancreatic Cancer;

Introduction

We had previously demonstrated that we could incorporate gemcitabine molecules into the backbone of an siRNA targeting CHK1 and studied this drugs effects in pancreatic cancer cells [1]. Work by others demonstrated that silencing or inhibiting CHK1 could augment the activity of gemcitabine (2'-deoxy-2',2'-difluorocytidine monohydrochloride) in a number of tumor cell types [2-4]. Gemcitabine is a pyrimidine-based nucleoside that acts as a cytidine analog, where two fluorine atoms have replaced the hydroxyl on the ribose [5]. When administered systemically, it is taken up by nucleoside transporters, activated by triphosphorylation (by deoxycytidine kinase) and can then be incorporated into either RNA or DNA. It replaces the nucleotide Cytidine during DNA replication [5] and can inhibit tumor growth since new nucleotides cannot be attached to this nucleotide mimic, resulting in apoptosis of the cells [1]. Gemcitabine is an approved therapeutic for treating various cancers including pancreatic cancer [6]. It acts as a nucleoside metabolic inhibitor that causes DNA damage and blocks the progression of cells through the G1/S phase boundary, however, it has poor bioavailability and, when used in patients, requires infusion in large doses, resulting in significant toxicity [7]. Checkpoint kinase 1 (CHK1) is an integral part of DNA repair and also regulates G1/S transition [8,9]. Inhibition of CHK1 may enhance sensitization to DNA-damaging agents via downregulating ribonucleotide reductase levels, shown to be important for resistance to gemcitabine activity [10]. Ribonucleotide reductase is composed of the homodimeric RRM1 and RRM2 subunits that catalyze the conversion of ribonucleotides to deoxyribonucleotides (dNTs). These are used in the synthesis of DNA during replication and repair. Consequently, CHK1 inhibition results in exhaustion of dNTs and enhanced DNA damage [4]. A small molecule inhibitor and an siRNA targeting CHK1 decreased RRM1 and 2 and increased gH2AX, an established biomarker for DNA double-strand breaks (also increased by gemcitabine treatment) [1, 4]. CHK1 inhibition can increase the cytotoxicity of gemcitabine by interfering with DNA damage checkpoints independent of p53 status in pancreatic cell lines [4].

We previously reported that we could directly incorporate gemcitabine as a modified nucleotide into the sequence of an oligonucleotide encoding an siRNA sequence against CHK1 and these constructs demonstrated improved efficacy of the product in pancreatic tumor cells compared to either treatment alone [1]. Specifically, we identified that we could substitute gemcitabine in place of Cytidines in the siRNA sense strand and demonstrated that two gemcitabines per sense strand along with two at the 3' end of the antisense strand gave optimal synergistic effect in reducing the cell viability of pancreatic tumor cells in vitro [1]. CHK1-gemcitabine constructs retained the activity of gemcitabine (inducing RRM1 and RRM2 activity) and CHK1 inhibition (both at the gene and protein level) and induced gamma H2AX through silencing of CHK1 [1]. The transfection of this siRNA construct into pancreatic tumor cells (using lipofectamine) produced a synergistic effect on cell killing by the combination not seen with either CHK1 siRNA or gemcitabine alone and the constructs demonstrated improved potency and efficacy [1].

The constructs reported previously were synthesized using native bases (chemically unmodified). It has been shown that chemically modifying the bases (using 2'-Fluoro or 2'-Methoxy modifications) in an siRNA can reduce off target effects and can also result in prolonged stability of the siRNA with longer silencing effects [11]. We explored the effect of chemically modifying the siRNA backbone on the ability of the construct to act compared with the unmodified construct and we found that the products demonstrated a further increase in potency in all cells tested. We further explored the utility of the constructs to inhibit other tumor types including ovarian, triple negative breast cancer (TNBC) as well as lung cancer and the product demonstrated improved efficacy and potency in all cell types studied.

Finally, we formulated a construct, that showed potent activity in vitro (transfected with Lipofectamine RNAiMax), with polypeptide nanoparticles (consisting of HKP(+H) – previously shown to deliver siRNAs to tumors in vivo) [12] and further tested the nanoparticle constructs in vivo using a pancreatic tumor xenograft model. The product demonstrated inhibitory effects in vivo with no effect on the body weights of the animals. This formulation may prove useful as a therapeutic to treat cancer.

Materials and Methods

Cell Lines

All cell lines were obtained from ATCC (Rockville, MD). Cells were cultured in standard media supplemented with 10% FBS: Panc 10.05, H1299 and H358 in RPMI-1640 Medium, MiaPaCa-2, HT29 and MDA-MB-231 in DMEM, LS180 in EMEM. All media were obtained from ATCC (Rockville, MD).

Cell Viability Assay

Cells were seeded in 384-well plates at a density of $0.3x10^3$ to $1x10^3$ cells/well. On the next day, cells were treated with serially diluted gemcitabine or siRNAs delivered by Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA). 96 hours after addition of gemcitabine or transfection reagent, the number of viable cells was determined using CellTiter-Glo(R)-2.0 reagent (Promega, Madison, WI) by measuring luminescent signal on a Cytation 5 plate reader (BioTek Inc, Winooski, VT). All values were compared to values generated for cells treated with non-silencing (NS) siRNA or to non-treated control (100%) and reported as the percentage of this value. Values represent the mean ±SD (n=4) in each experiment and experiments were repeated two more times with similar results.

Calculation of EC50 Values

The EC50 values of the Gemcitabine, or Gemcitabine-modified siRNAs, (half-maximal effective concentration) were derived from a sigmoidal dose-response (variable slope) curve using GraphPad Prism 10.0.2(232) software (GraphPad Software, San Diego, CA).

Combination Index (CI) Calculation

Synergism, additivity or antagonism in the different combinations was calculated on the basis of the multiple drug effect equation and quantitated by the combination index (CI) [38], where CI = 1 indicates that the two drugs have additive effects, CI<1 indicates more than additive effects (''synergism'') and CI>1 indicates less than additive effects (''antagonism'').

CI = (D)1/(Dx)1+(D)2/(Dx)2+(D)1(D)2/ (Dx)1(Dx)2, where (Dx)1 and (Dx)2 are the doses of drug 1 and drug 2, alone, inhibiting 50%, whereas (D1) is the dose of drug 1 in combination, and (D2) the dose of drug 2 in combination that gives the experimentally observed 50% inhibition.

Serum Stability Assay

The stability of the siRNAs was confirmed using polyacrylamide gel electrophoresis. The gemcitabine containing siRNAs were incubated with 90 % (v/v) human serum for different time points at 37°C, and a sample was collected at 1, 4 and 18 hours. The siRNA without human serum was used as a control (0 h). After mixing the loading dye, mixtures of the siRNA and serum were loaded into 15 % native polyacrylamide gel with 1.0 X TBE. The PAGE gel was run for 60 min at 150 V. The gemcitabine-siRNAs in serum were analyzed on a gel imaging system (Azure 400, Azure Biosystems).

Manufacture of siRNAs

RNAs were synthesized with DMT ON using standard RNA synthesis protocols and commercially available reagents on a Mermade 12 oligo synthesizer. Dimethylformamidine (dmf) protected G-phosphoramidites were utilized for the synthesis of gemcitabine-containing RNAs, facilitating milder cleavage and deprotection conditions. Cleavage of gemcitabine containing RNAs was completed at room temperature using 1 mL 2N ammonium hydroxide in methanol for 2 hours. Subsequently, 1 mL 28-30% ammonium hydroxide solution was added and cleaved RNAs were incubated at 45 °C for 16 hours to ensure complete deprotection of exo-amine groups. For RNAs without gemcitabine, cleavage and deprotection were achieved simultaneously using an AMA solution (1:1 28-30% ammonium hydroxide: methylamine) at room temperature for 2.5 hours. Purification of both gemcitabine and non-gemcitabine containing RNAs was carried out following the Glen-pack DNA purification cartridge protocol, ensuring purity levels surpassing 85%. RNA purity was assessed using an anion exchange HPLC, and the identity of all single strands was confirmed via HRMS analysis. RNAs containing phosphorothioate linkages tend to produce diastereomers of the same RNA which could be seen as multiple peaks in HPLC analysis for such RNAs, but they result in a single most abundant mass peak. Quantification of RNAs was performed using a UV-visible spectrophotometer. siRNA constructs were prepared in RNase-free water by heating sense and antisense strands in equimolar ratios at 90 °C for 5 minutes, followed by gradual cooling to room temperature over 2 hours. These meticulous syntheses, cleavage, deprotection, and purification techniques were employed to ensure the high quality and integrity of the RNA constructs, making them suitable for biological investigations. Detailed information on syntheses, along with quality control (QC) validation results, are available in the Supplemental Materials.

Formulation of Nanoparticles

Peptide nanoparticle (PNP) formulations were prepared using a microfluidic mixing system with a staggered herringbone patterned mixer (Precision NanoSystems, Vancouver, BC, Canada). HKP(+H) stock was prepared in water and diluted to appropriate concentrations. PolyGem siRNA or non-silencing (NS) siRNA stocks were prepared in water. The HKP(+H) and siRNA were mixed at a volume ratio of 1:1 at 8-10 mL/min total flow rate. Formulations were incubated for 30 min at room temperature.

Particle size and zeta potential were then determined by dynamic light scattering with a Zetasizer Ultra (Malvern Panalytical, MA). A gel retardation assay was used to evaluate the efficiency of complex formation. Briefly, pre-formed PNPs were mixed with RNA loading dye and applied to 2 % agarose gel. Gel was run in 0.5 X TBE for 15 min. Electrophoretic mobility of the siRNA-nanoparticles was analyzed on a gel imaging system (Azure 400, Azure Biosystems).

In Vivo Assays

In vivo studies were performed at Crown Bio San Diego. Eight female NOD/SCID mice from Jackson labs were used per group. MiaPaca2 cells were cultured and, at time zero, 3x10e6 cells were inoculated as xenografts on the backs of mice in 0.1ml of 1:1 PBS:Matrigel. When tumors reached ~200mm3, mice were randomized based on tumor volume and treated, by IV injection, with either control (Non-silencing siRNA in PNP at 2mgs/kg) or with test agent (PGN6 siRNA in PNP at 2mgs/kg). Treatment was then provided twice weekly (BIW) throughout the experiment. Body weight and tumor volume were each recorded twice per week. After 6 doses were administered BIW, treatment was halted but measurement continued for the next week.

Results

We synthesized the siRNA sequences shown (Table 1) against CHK1 and we evaluated several different chemical modification patterns of the siRNA backbone - varying the locations of the 2'-Fluoro bases relative to the 2'OMe modifications and the incorporation of gemcitabine. Various constructs were examined where the 2 gemcitabines in the sense strand were augmented by inclusion of an additional 2 gemcitabines at the 3' end of the AS strand (PGN6, PGN7 and PGN7S). PGN7S also contained phosphorothioate groups at the ends of each strand (Table 1). We tested these siRNA constructs in vitro for their ability to reduce viability of pancreatic cancer using Miapaca2 cells after lipofectamine transfection of the siRNAs at varying concentrations (Fig. 1). All of the constructs could inhibit the viability of these cells by 100% at concentrations below 100nM. We further examined the contribution of the gemcitabine within the constructs by making constructs lacking the gemcitabine ('PGN6 no gem' and 'PGN7 no gem'; Fig. 1). As expected, the sequences lacking gemcitabine were much less effective in MiaPaca2 cells (and in all cells tested). The initial CHK1 construct lacking chemical modifications (CHK1-21-wt) showed a similar IC50 value to the modified siRNAs lacking gemcitabine (PGN6 no gem and PGN7 no gem; Fig. 1), but showed reduced efficacy at higher doses, producing only a partial inhibition of cell viability at the higher concentrations. The modified siRNA lacking the gemcitabine, (PGN6 no gem and PGN7 no gem), showed slightly greater inhibition at higher concentrations (Fig. 1). The dose response curves for PGN6 and PGN7 in MiaPaca2 cells overlapped and showed a 100-fold improvement over gemcitabine alone (Fig. 1).

Name	Sense Strand	Antisense strand
Non-Silencing	5'-CGAGCAGGGUAUCGACGAUUACAAA-3'	5'-UUUGUAAUCGUCGAUACCCUGCUCG-3'
PGN6	**5'-AAGAAAgAgau[GEM]UGUAU[GEM]AAU-3'**	**5'-AuUGAuACAGAUCuCuuucuu[GEM][GEM]dTdT-3'**
PGN7	**5'-AAGAAAgAgau[GEM]uGuAu[GEM]Aau-3'**	**5'-AuUGAuACAGAuCuCuUuCuU[GEM][GEM]dTdT-3'**
PGN6 (no Gem)	**5'-AAGAAAgAgaucUGUAUcAAU-3'**	**5'-AuUGAuACAGAUCuCuuucuudTdT-3'**
PGN7 (no Gem)	**5'-AAGAAAgAgaucuGuAucAAu-3'**	**5'-AuUGAuACAGAuCuCuUuCuUdTdT-3'**
PGN7S	**5'-A*A*GAAAgAgau[GEM]uGuAu[GEM]A*A*u-3'**	**5'-Phos-A*u*UGAuACAGAuCuCuUuC*u*U[GEM][GEM]dTdT-3'**
Normal capital letters are unmodified bases, Bold uppercase letters are 2'-OMe modified bases, bold small case letters are 2'-Fluoro modified bases; [GEM] – gemcitabine nucleosides; *=Phosphorothioate; Phos=phosphate. Note CHK1-21-wt sequence is the same as PGN6 (no Gem) but the siRNA is not chemically modified.		

Table 1: The Constructs Tested.

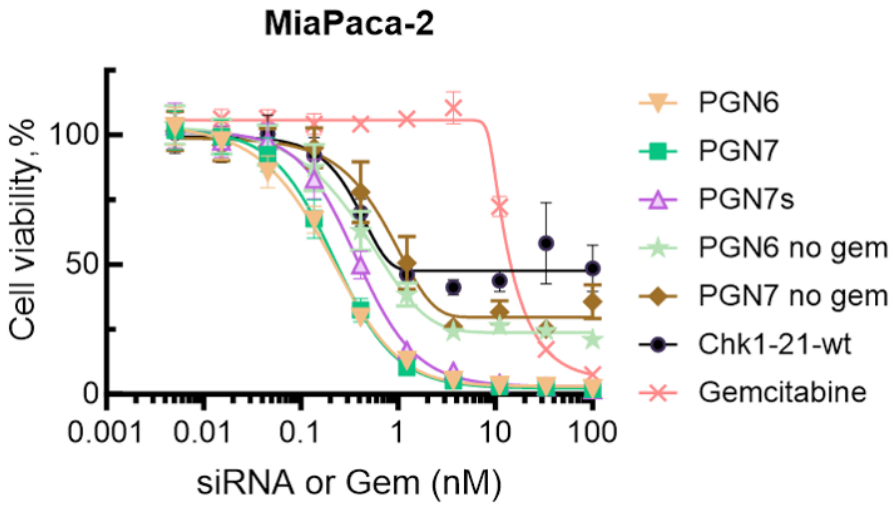


Figure 1: Effect of chemical modification and Gemcitabine incorporation within the CHK1 siRNA on viability of MiaPaCa-2 cells.

MiaPaca2 Cells were seeded at $0.3x10^3$ cells/well and incubated for 96 hours post-transfection, the number of viable cells was determined with CellTiter-Glo(R)-2.0 reagent (Promega, Madison, WI) by measuring luminescent signal using a Cytation 5 plate reader (BioTek Inc, Winooski, VT). All values were normalized to values generated for cells treated with a non-silencing siRNA and reported as the percentage cell viability. Values represent the mean ±SD (n=4). The results are representative of 3 experiments.

We noted that the unmodified CHK1 siRNA sequence (containing native bases without gemcitabines) showed a melting temperature (Tm) for the duplex (separating into SS and AS strand) of 65^0C (Table 2). Incorporation of 2 gemcitabines into the unmodified sense strand alone (construct STD1) gave an increase in Tm to 71.5^0C. Modification with 2'F and 2'OMe bases increased the Tm of the constructs significantly in sequences lacking gemcitabine (PGN6 no gem = 81.6^0C and PGN7 no gem = 80.1^0C). Adding the 2 gemcitabines in the sense strand (in place of the Cytidine bases) and the 2 gemcitabines at the end of the AS strand decreased the Tm to 74.6^0C for PGN6 and 74^0C for PGN7 (Table 2). This reduction in Tm for the gemcitabine containing constructs may explain the more potent action of the modified constructs compared to the unmodified siRNA alone (CHK1-21-WT).

Name	Tm	Reference Figures
CHK1-21-WT	65.0 °C	Figure SI-OS- 33 and 34
STD1	71.5 °C	Figure SI-OS- 31 and 32
PGN6 (no Gem)	81.6 °C	Figure SI-OS- 25 and 26
PGN7 (no Gem)	80.1 °C	Figure SI-OS- 27 and 28
PGN6	74.6 °C	Figure SI-OS- 21 and 22
PGN7	74.0 °C	Figure SI-OS- 23 and 24
PGN7S	73.6 °C	Figure SI-OS-29 and 30

Table 2: Melting Temperatures of the Duplexes.

Melting temperatures (Tm) were measured as described in Supplemental Information and shown in the Supplemental Figures as indicated above. STD1 is the same sequence as PGN6 but the siRNA is not chemically modified and only contains 2 gemcitabines in the Sense strand. CHK1-21-wt sequence is the same as PGN6 (no Gem) but the siRNA is not chemically modified.

We further examined the effect of combining the modified CHK1 siRNA containing gemcitabine (CHK1-Gem siRNA) with other siRNAs against targets that, when silenced, were expected to augment the activity of the CHK1-Gem siRNA.

Rationale for Combining CHK1-Gem siRNA with WEE1 siRNA

In our previously published work [1] we showed that CHK1-Gem siRNA demonstrated additivity with an siRNA against a second target (WEE1) in treating MiaPaca2 and BxPC3 pancreatic cells. WEE1 has been shown to augment activity of CHK1 inhibition [8, 13-15] and be a relevant therapeutic target for cancer in its own right [16]. WEE1 regulates the G2/M checkpoint by catalyzing an inhibitory tyrosine phosphorylation of CDK2/cyclin B kinase complex and hence terminates the cell cycle. Inhibiting WEE1 has been shown to lead to DNA damage due to unchecked replication and potentiates the effect of DNA-damaging therapeutics [16]. WEE1 is downstream of CHK1 and therefore WEE1 siRNA will

also increase the activity of gemcitabine. Consequently, inhibition of WEE1 using siRNA is expected to have a further antitumor effect – especially in the presence of gemcitabine and inhibition of CHK1 - and possibly without a concomitant increase in in side effects or toxicity in normal tissues [13,15,17]. We extended on our previous data and showed that CHK1-Gem siRNA augmented the activity of WEE1 siRNA in a number of cell lines. This included pancreatic cancer cells, lung cancer cells and colon cancer cells (Fig. 2a).

Cells were seeded at $0.4x10^3$ cells/well and cells were transfected with siRNAs indicated and incubated for 96 hours. SiRNAs used were PGN7 + non-silencing siRNA (PGN7 + NS), WEE1 siRNA + non-silencing siRNA (WEE1 + NS) or PGN7 + WEE1 siRNA. The number of viable cells was determined with CellTiter-Glo(R)-2.0 reagent (Promega, Madison, WI) by measuring luminescent signal using a Cytation 5 plate reader (BioTek Inc, Winooski, VT). All values were normalized to values generated for cells treated with a non-silencing siRNA transfected using the same conditions and reported as the percentage cell viability. Values represent the mean ±SD (n=4) from a representative experiment repeated twice more. Synergy was determined by calculating the Combination Indexes (CI) for each figure below (as described in Methods) and the following values were obtained:

Cell line	CI	Effect
H358	0.83	Synergism
Panc10.05	0.7	Synergism
H1299	0.69	Synergism
MiaPaca2	0.39	Synergism
LS180	0.33	Synergism
A CI<1 indicates more than additive effects (“synergism”)		

2A

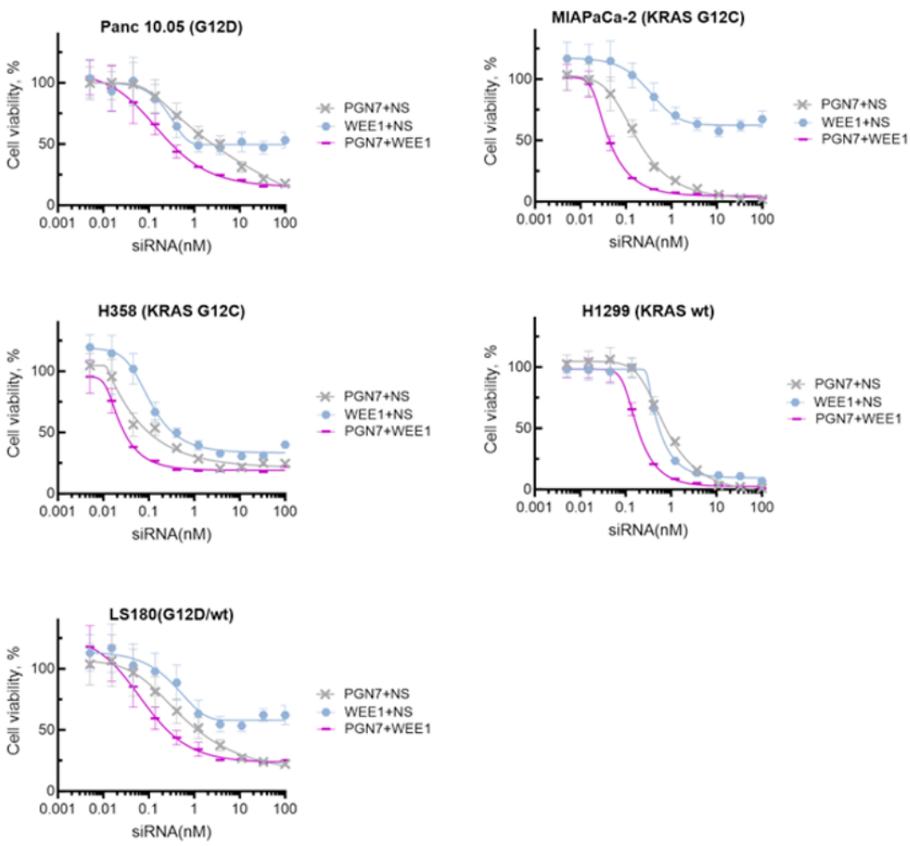


Figure 2A: Effect of concurrent treatment with PGN7 and WEE1 siRNA on cell viability.

In all cell lines, CHK1-gemcitabine siRNA and WEE1 siRNA provided an increase in potency, compared to either siRNA alone (Fig. 2a), and showed synergy as determined by the Combination Index (<1). H358 (CI= 0.83), Panc10.05 (CI=0.7), H1299 (CI= 0.69), MiaPaca2 (CI= 0.39) and LS180 (CI= 0.33).

Rationale for Combining CHK1-Gem siRNA with Bcl-xL siRNA

A previous study [18] demonstrated that in 10 cell lines derived from different cancers, high Bcl-2 baseline expression was observed in cell lines that were resistant to gemcitabine (GEM-R). Gossypol treatment resulted in the decrease of anti-apoptotic genes such as Bcl-2 and Bcl-xL and an upregulation of the pro-apoptotic gene, Noxa, and demonstrated synergism with Gemcitabine in these tumor cells.

We found that inhibition of Bcl-xL by siRNA was selective in augmenting the activity of CHK1-gemcitabine siRNA in the various cell lines studied (Fig. 2b). The combination increased efficacy in Panc10.05 cells, had limited effect in H358 and LS180 cells (CI~1),

but had little effect in MiaPaca2 cells or H1299 as determined from the Combination Index that suggested synergy of the combination in Panc10.05 (CI=0.28), additivity in H358 (CI = 0.95) and in LS180 (CI= 1.09), and showed no additivity in MiaPaca2 or H1299.

The experiment was performed as detailed in Fig. 2A but an siRNA against Bcl-xL replaced the WEE1 siRNA. Values represent the mean ±SD (n=4) from an experiment repeated three times. All values were normalized to values generated for cells treated with a non-silencing siRNA transfected using the same conditions and reported as the percentage cell viability.

Combination Index (CI) was calculated as in Methods.

Cell line	CI	Effect
LS180	1.09	Additivity
H358	0.95	Additivity
Panc10.05	0.28	Synergism
A CI<1 indicates more than additive effects (“synergism”), a CI>1 indicates less than additive effects (“antagonism”), a CI of ~1 suggests additivity.		

2B

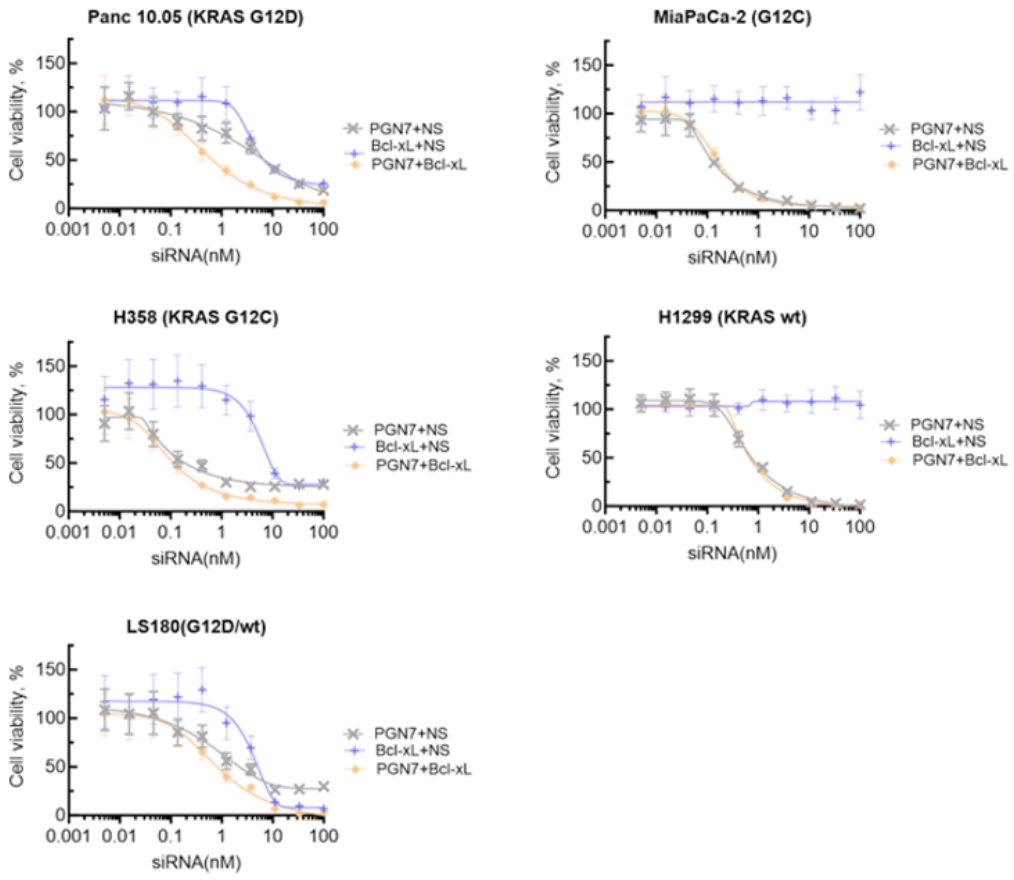


Figure 2B: Effect of concurrent treatment with PGN7 and Bcl-xl siRNA on cell viability.

Efficacy of CHK1-Gemcitabine Constructs in TNBC and Ovarian Cancer Cells

In TNBC (MDA-MB-231) cells and ovarian cancer cells (Ovcar3) we saw that the chemically modified CHK1-21 sequences (lacking gemcitabines in the construct) were noticeably less effective than constructs containing gemcitabine. CHK1-gemcitabine siRNAs (PGN6 and PGN7) exhibited similar but increased potency in both cell types (Fig. 2c).

MDA-MB-231 cells (TNBC) or Ovcar-3 cells (ovarian cancer) were seeded at $0.6x10^3$ cells/well and incubated for 96 hours with lipofectamine and the reagents shown in the figure. 'PGN6/7 no gem' refers to the construct without gemcitabine. The number of viable cells was determined with CellTiter-Glo(R)-2.0 reagent (Promega, Madison, WI) by measuring luminescent signal using a Cytation 5 plate reader (BioTek Inc, Winooski, VT). All values were normalized to values generated for cells treated with a non-silencing siRNA and reported as the percentage cell viability. Values represent the mean ±SD (n=4).

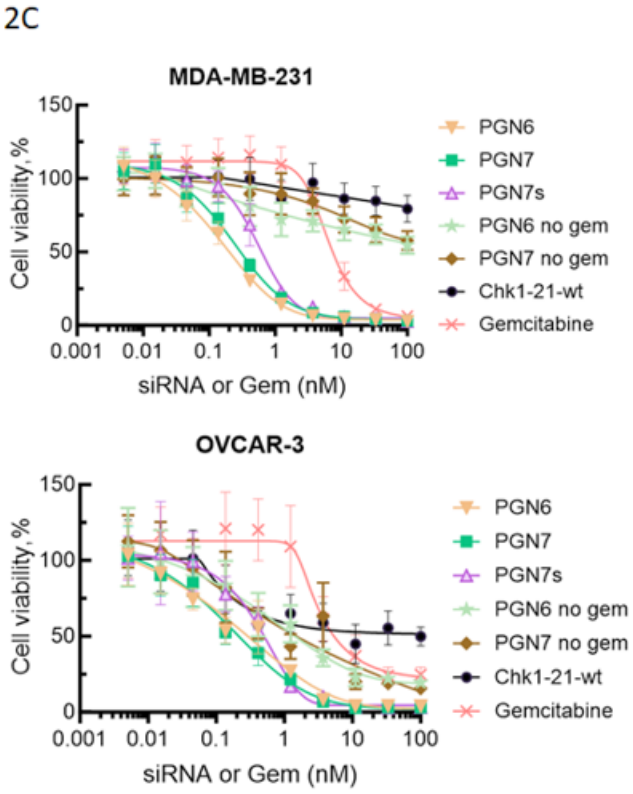


Figure 2C: Effect of various siRNA constructs on viability of TNBC and ovarian tumor cells.

Manufacture of Nanoparticles for Delivery in Vivo

To be able to test the efficacy of the products in vivo we formulated siRNAs (non-Silencing siRNA (Control; NS), and CHK1-gemcitabine siRNA (PGN6)) with a branched histidine lysine polymer (HKP (+H)) to form nanoparticles (Fig. 3). The nanoparticles formed showed size characteristics between 82.6nM (for NS) and 86.7nM (for CHK1-gemcitabine siRNA) and uniformity, as determined by low polydispersity index (PDI), using dynamic light scattering in a Malvern/Wyatt instrument. They also showed weakly positive charge (zeta potential of 34mV for the non-silencing siRNA formulated in HKP(+H) and 35.8mV for CHK1-gemcitabine siRNA in the same particles).

A.

PNP Formulation	Size (nm)	PDI	Zeta potential (mV)
PGN6	86.7 ± 0.54	0.115	35.6
NS	82.6 ± 2.72	0.045	34.8

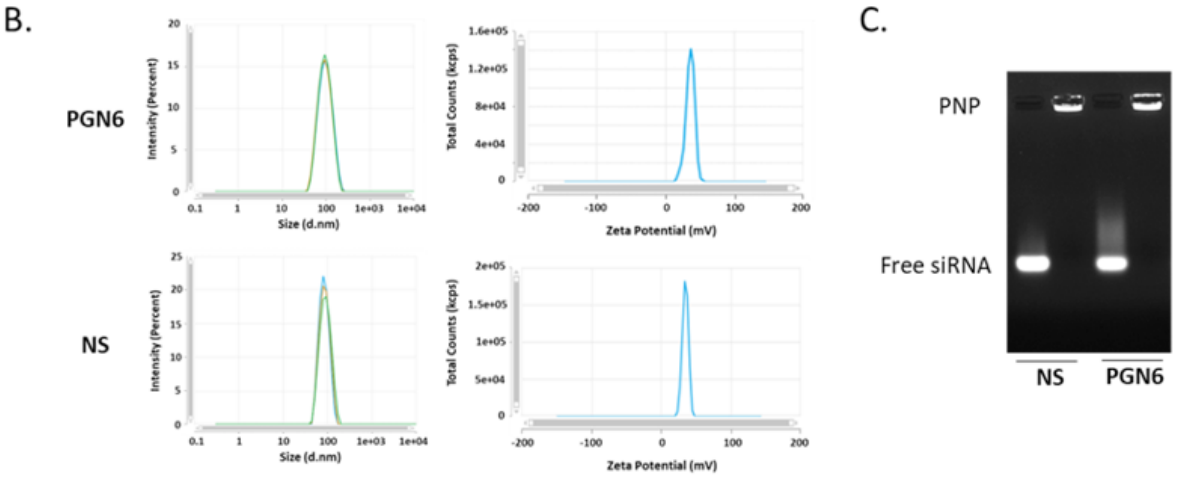


Figure 3: Characterization of PNP formulations with PGN6 or NS siRNA.

Each siRNA was mixed with HKP(+H) in aqueous solution at a ratio of 3:1 (w/w) using a PNI Nanoassemblr microfluidic mixer at 8 ml/min to form nanoparticles.

A) After incubation for 30 min, the size of the PNP formulations were measured using dynamic light scattering. Zeta potential was assessed using a Zetasizer Ultra (Malvern Panalytical, MA).

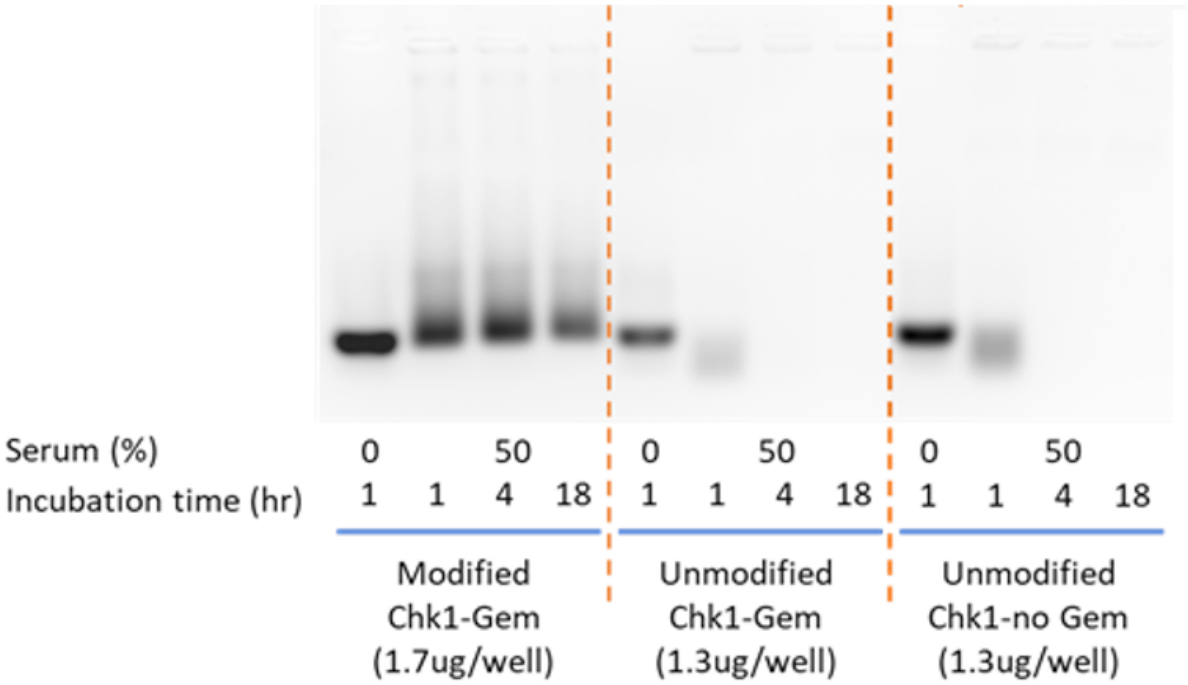
B) Representative particle size distribution and zeta potential of PGN6 and NS PNP formulations.

C) The PNP/siRNA formulation was checked using a gel retardation assay for siRNA formulated with HKP(+H). Nanoparticles mixed with a 6 X loading dye were loaded into a 2 % agarose gel followed by electrophoresis at 100 V for 15 min, and the agarose gel was visualized using an Azure gel imaging system.

We further explored the sensitivity of the siRNAs to nucleases present in serum (Fig. 4a). The chemically modified siRNA were protected from degradation. They were stable for 18h at 37^0C when treated with 50% serum (Fig. 4A upper panel). This contrasts with the unmodified siRNAs that showed rapid degradation by 50% serum (no siRNA was visible after a 4h incubation).

We further compared modified CHK1-gemcitabine siRNAs with unmodified CHK1 siRNA after exposure to 90% serum (Fig. 4a lower panel). The 2'F and 2'OMe modifications in the backbone of the siRNA protected the siRNA from serum while most of the unmodified siRNA was degraded in a little over an hour (Fig. 4a lower panel).

Upper Panel: The stability of chemically modified and unmodified CHK1-Gem siRNA was tested in human serum. This will mimic exposure in vivo. SiRNAs were exposed to 0% or 50% human serum for the time indicated in the figure. After exposure, the siRNAs were assessed using native PAGE analysis (upper panels).



Lower Panel: Shows various siRNA constructs after exposure to 90% human serum for the times indicated before running on a gel to show integrity of unmodified siRNA targeting CHK1 (left) and modified siRNA with or without gemcitabine.

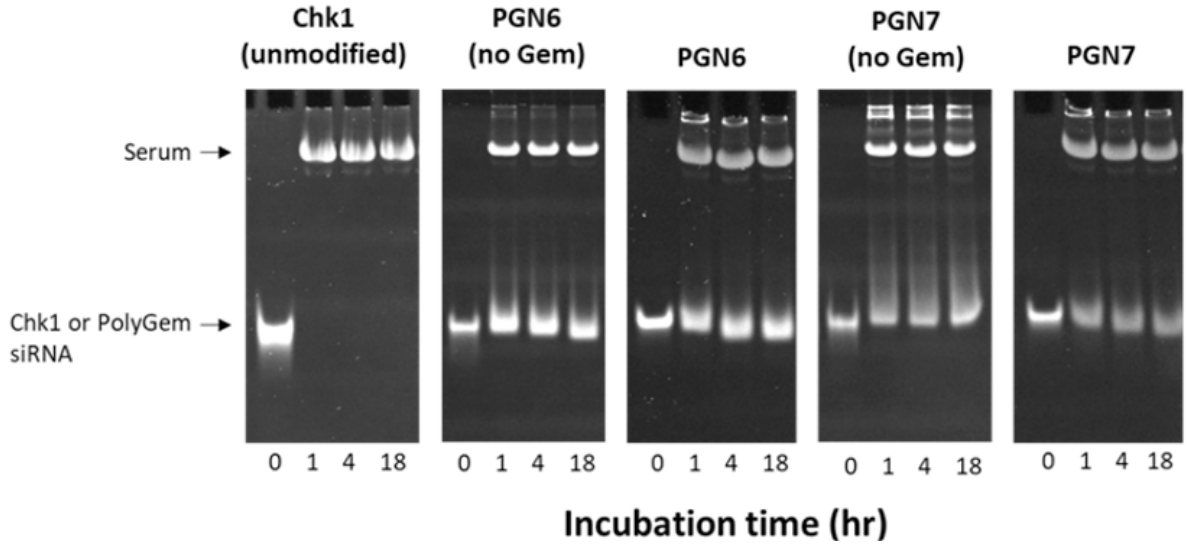


Figure 4A: Stability of chemically modified and unmodified CHK1-Gem siRNA in Serum.

We also observed time and dose-dependent killing of MiaPaca2 cells and MD-MB-231 cells in vitro when PGN6 was formulated with HKP(+H) to create nanoparticles (with the characteristics shown in Fig. 3) and these were used to transfect the cells (Fig. 4b). The apparent potency was reduced because PNP may take longer to get siRNA into cells than lipofectamine, but we still observed ~5nM

IC50 for PNP delivered PGN6 at 96h in MiaPaca2 cells in vitro and a slightly higher IC50 for PNP delivered PGN6 in MDA-MB-231 cells in vitro.

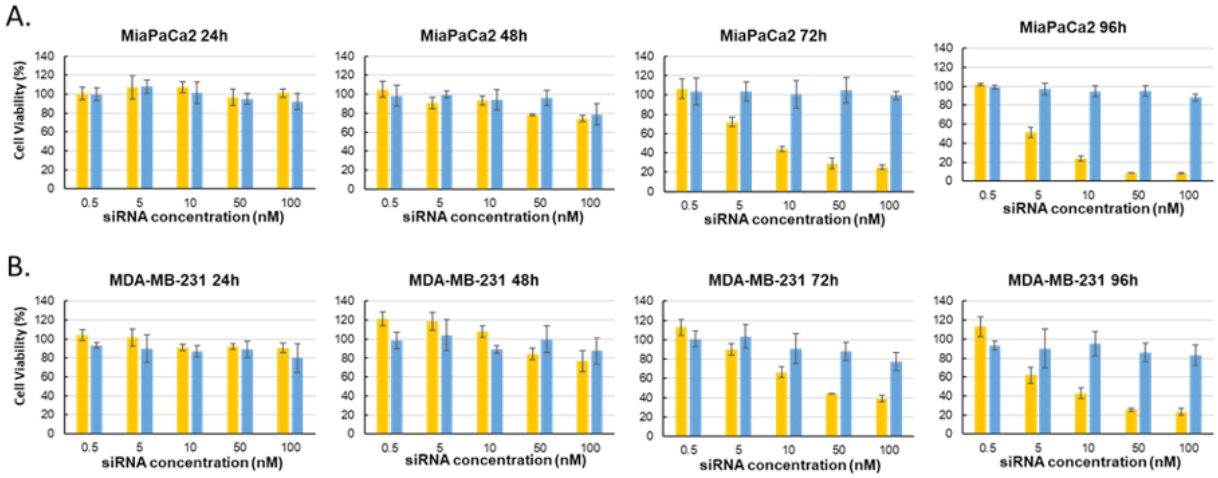


Figure 4B: Viability of MDA-MB-231 and MiaPaca2 cells upon exposure to PGN6 formulated in nanoparticles of HKP(+H) (PNP) for various times.

Viability of A) MiaPaca2 cells and B) MDA-MB-231 cells after incubation with PGN6 in nanoparticles of PNP (Left most bars) or non-silencing siRNA in nanoparticles of PNP (Right most bars) after exposure for 24-96 h. Cells were seeded at $0.4x10^3$ cells/well. Cell viability was determined by CellTiter-Glo cell viability assay at the conclusion of the incubation times shown in the figure. Values represent the mean ±SD (n=4).

We then explored the ability of nanoparticles to deliver the modified siRNAs to a xenograft tumor when administered IV (Fig. 5; left panel). In this experiment PGN6 or NS siRNA were formulated with HKP(+H) as in Fig. 3 and were administered for 3 weeks BIW (2x per week) by intravenous administration in mice bearing a MiaPaca2 xenograft tumor. Administration of the products began after the tumor volume first reached $200mm^3$. Tumor growth was monitored before every administration. PGN6 resulted in significant inhibition of tumor growth from the day of first administration. Dosing was halted at day 18 and the tumor was monitored for an additional week. No regrowth of the tumor was observed at this time (Fig. 5; left panel). Treatment with PGN6 formulated in HKP(+H) had no effect on body weights of the treated animals suggesting no significant toxicity of the formulation (Fig. 5; right panel).

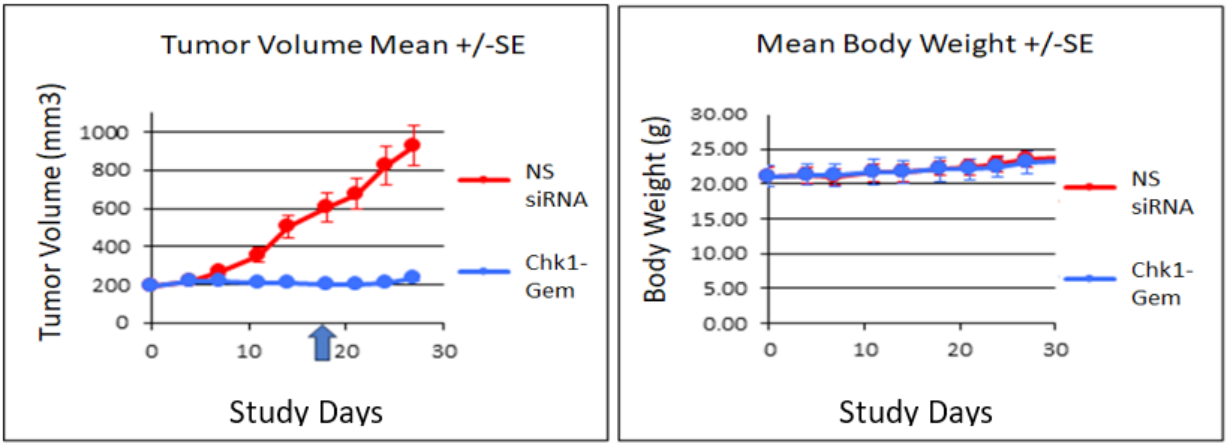


Figure 5: Effect of HKP(+H) (PNP) formulated with chemically modified CHK1 siRNA containing gemcitabine (PGN6) on MiaPaca2 xenografts in mice.

All animal studies were performed at Crown Bio (San Diego) using NOD/SCID mice. Eight animals per group were randomized into 2 groups based on xenograft size after implantation and treated with either PGN6 formulated in nanoparticles of PNP (HKP+H; blue lines in figure) or with Non-silencing siRNA in the same PNP (Control; red lines in figure). Mice were treated with 2mgs/kg of each product administered IV BIW when the xenograft reached 200mm^3. A total of 6 doses were administered and tumor size and animal body weights were measured twice a week. Animals were followed for an additional week after the last dose (indicated by the arrow). Data are presented as the mean +/-SEM for each data point from 8 animals.

Discussion

Rationale for Chemically Modified Bases in siRNA

Oligonucleotide therapeutics, such as those based on small interfering RNA (siRNA), are promising agents against pancreatic cancer, because they can identify a specific mRNA sequence and interfere with gene expression as molecular-targeted agents [19]. Consequently, RNAi therapeutics have been formulated with nanocarriers to treat pancreatic tumors [20].

Work by Azorsa [2] and Fredebohm [3] had demonstrated that small molecule inhibitors of CHK1 or siRNA targeting CHK1 could synergize with the concomitant addition of free gemcitabine against pancreatic tumor cells in vitro. We previously showed that incorporating gemcitabine nucleotides into the sequence of a chemically unmodified siRNA targeting CHK1 augmented the antitumor activity observed in vitro against various pancreatic cell lines [1]. We demonstrated that maximal activity, against various pancreatic tumor cells in vitro, was observed when 4 gemcitabine molecules were incorporated into the siRNA: 2 within the sense strand (gemcitabine replacing cytidines) and 2 at the 3' end of the antisense strand [1]. The combination retained efficacy of the siRNA (shown by western blot to be able to reduce the amount of CHK1 protein [1]), whilst also showing the efficacy of gemcitabine (increasing RRM1 and RRM2 proteins in western blots [1]), and the combination showed synergistic effects in vitro by destabilizing the DNA replication apparatus [4, 8].

There are a number of reasons for incorporation of chemically modified bases into siRNA; It can improve half-life of the product in serum (as shown here (Fig. 4a)) and can improve efficacy against the target while minimizing off target effects [11]. In this paper we furthered our earlier work [1] by using chemical modifications of the CHK1 siRNA backbone (specifically incorporating 2'-Fluoro and 2' O-Methyl nucleotides) to enhance its stability. We retained the same nucleotide sequence in the siRNA targeting CHK1 as previously published but varied the locations of 2'F and 2'OMe modified bases in 2 constructs: PGN6 and PGN7 (Table 1). 2'OMe modification of the ribose provides a bulkier option than 2'F modification and provides better resistance to degradation by nucleases [21]. Too many of the natural base 2'OMe modifications however can diminish siRNA activity against its target, but the modifications can also suppress siRNA-driven innate immune activation, enhance specificity for the target (increasing activity), and reduce toxicity due to off-target mediated effects [22]. 2'OMe may play a role in enhancing stability and increases binding to mRNA [22].

We didn't perform a thorough evaluation of base changes but made rational changes to the siRNA to incorporate these modifications. For example, we selected bases 9-11 on the sense strand to be modified with 2'F (since this corresponds to the cleavage location of the cognate mRNA [21,23]). We also made the 14th base in the antisense strand a 2'F since this position does not tolerate 2'OMe changes [24]. We further compared changes in the number of 2'F bases (with higher numbers in PGN6 than in PGN7). However, while the siRNA could be sensitive to the location of these changes, PGN6 and PGN7 show very similar results in a number of the cell lines tested, so, besides stabilizing the construct against serum nucleases (Fig. 4A), the two constructs also demonstrated very similar melting temperatures (Table 2).

We also examined the modified siRNA and showed that adding gemcitabines improved the potency of the siRNA - presumably through additive effects of silencing CHK1 and the activity of released gemcitabine (as shown separately by Azorsa, Fredebohm and Liang [2-4]). Another possibility for this behavior is that hybridization between gemcitabine (incorporated in place of Cytidine) in the sequence on the sense strand and the corresponding Guanidine on the AS strand is weaker and decreases the melt temperature (Table 2). This allows more ready separation of the 2 strands and may facilitate the binding of the AS strand to the RISC complex.

CHK1-21-WT was not chemically modified, and did not contain gemcitabine, and it was not as potent as PGN6 or PGN7 (each containing four gemcitabines) in any of the cells tested (Figs. 1 and 2c). PGN6/7 without gemcitabine (PGN6/7 no gem) also showed reduced efficacy compared to the same molecules with gemcitabine, and these 2 constructs gave similar results to CHK1-21-WT (Figs. 1 and 2c).

Incorporation of phosphorothioates can further stabilize siRNA and protect the molecules from nucleases [22]. However, PGN7s (that also had Phosphorothioates (PS) at each end of the SS and AS strand) showed weaker activity than PGN7 (without PS). This reduction may be due to slower degradation of the molecule, (to release the gemcitabines), or due to slower separation of the strands to release the antisense strand and silence CHK1. As stated above, and shown in Table 2, the Tm of PGN7S is very similar to

PGN7, so this may not be the explanation. The PS modification also increases hydrophobicity of the siRNA, allowing interaction with plasma proteins [22]. However, in this study, we used a branched polypeptide (HKP(+H)) that spontaneously forms nanoparticles about 80nM in diameter around the siRNA. This nanoparticle protects the siRNA from interactions with plasma proteins and nucleases in vivo and may obscure any benefit of PS incorporation into the siRNA (Fig. 4A).

Rationale for Gemcitabine as a Therapeutic

Gemcitabine remains a cornerstone of PDAC treatment in all stages of the disease [6,25]. Despite suboptimal clinical effects primarily caused by molecular mechanisms limiting its cellular uptake, activation and efficacy, and the development of chemoresistance within weeks of treatment initiation, gemcitabine is also used to treat other cancers [26] such as non-small cell lung cancer [27], bladder cancer [28], ovarian [29] and breast cancer [30]. Gemcitabine alone can exhibit side effects such as low blood cell counts, hair loss, nausea, vomiting and myelosuppression [7,26,31]. Gemcitabine is hydrophilic and it is taken up into cells by nucleoside transporters [32]. However, repeated doses of Gemcitabine can result in drug resistance, presumably due to the dysfunction of these transporters required for cellular uptake [32]. Furthermore, it must be administered by infusion in order to able to get therapeutically relevant doses at the required tissue and to minimize toxicity. The ability to augment gemcitabine's activity by incorporation into siRNA targeting CHK1 that is delivered in vivo using nanoparticles (administered IV) will reduce systemic exposure to the drug and minimize its toxicity and drug resistance since nucleoside transporters will not be required for delivery via nanoparticles.

Notably, gemcitabine alone demonstrated much weaker activity than CHK1 siRNA gemcitabine constructs, giving an IC50 above 10nM in the in vitro studies using MiaPaca2 cells (Fig. 1).

Chemical modification of the siRNA backbone (using 2'F or 2'OMe) improved the serum stability of the construct over the unmodified sequence and reduced the rate of cleavage of gemcitabine from the constructs compared to the unmodified siRNA (Fig 4a). However, despite the variety of chemical modification patterns we tried (varying the position and number of 2'F and 2'OMe) we did not see a further improvement in potency. Indeed, PGN6 and PGN7 showed similar dose-response curves in all cells tested and therefore could be used interchangeably. TNBC cells (MDA-MB-231) and ovarian cancer cells showed improved potency and efficacy of CHK1-Gemcitabine siRNA constructs compared to either the siRNA alone or gemcitabine alone (Fig 2c).

Previous findings [33] also indicate that gemcitabine combinations could serve as a promising regimen for cancers other than pancreatic cancer. We studied the combination of gemcitabine incorporated into CHK1 siRNA in a variety of cancer cell lines. This included NSCLC with different KRAS backgrounds. H358 cells (KRAS G12C mutant cells) showed an IC50 of PGN7 of ~1nM (Fig. 2A) while other cell lines showed higher IC50 values (e.g. H2030 (G12C) showed an IC50 of 3nM; A549 (G12S) and H1299 (WT) showed an IC50 of ~6nM). In all cell lines, PGN7 and WEE1 siRNA provided an increase in potency, compared to either siRNA alone (Fig. 2a), and showed synergy as determined by the Combination Index (<1) in all cell lines. H358 (CI= 0.83), Panc10.05 (CI=0.7), H1299 (CI= 0.69), MiaPaca2 (CI= 0.39) and LS180 (CI= 0.33).

We also found that inhibition of Bcl-xL by siRNA was selective in augmenting the activity of PGN7 in the various cell lines studied (Fig. 2b). The Combination Index suggested synergy of the combination in Panc10.05 (CI=0.28), additivity in H358 (CI = 0.95) and in LS180 (CI= 1.09) and showed no additivity in MiaPaca2 or H1299.

CHK1-Gemcitabine siRNA Shows Activity Against a Tumor Xenograft in Vivo

Since we had demonstrated efficacy across a number of cell lines, we wanted to validate that the constructs could show similar efficacy when injected in a suitable model in vivo. We therefore used a mouse xenograft model of MiaPaca2 cells and formulated PGN6 (or non-silencing siRNA as a control) in a nanoparticle of HKP(+H). The products were injected IV through the tail vein of a mouse when the tumor reached a size of 200mm^3. The drugs were injected at 2mgs/kg BIW for 3 weeks and the results showed a significant inhibition in tumor growth by PGN6 (Fig. 5a) induced by the CHK1-Gemcitabine formulation, while animals treated with a control (non-silencing) siRNA formulated in the same nanoparticle showed growth of the tumor over time. CHK1-Gemcitabine nanoparticles showed no effect on body weights of the treated animals compared with the control treated animals (Fig. 5b) suggesting the therapeutic was well tolerated.

Conclusions

We demonstrated robust activity of a chemically modified siRNA backbone targeting CHK1 and containing gemcitabine in both the sense and antisense strands. The construct showed improved activity (compared with siRNA alone or gemcitabine alone) against a number of different tumor cell lines in vitro.

In this paper we pursued a nanoparticle formulation using a HKP polypeptide to protect the siRNA in vivo and to deliver multiple siRNAs per particle. HKP(+H) has shown the ability to protect the siRNA during IV administration, efficacy against tumor xenografts upon IV administration [12,34-37], and delivery of multiple siRNAs to a tissue [12].

The chemically modified construct delivered to pancreatic xenografts, using the polypeptide nanoparticle, demonstrated efficacy against the tumor. The inherent stability of the modified siRNA backbone, and resistance to degradation by serum, may allow direct conjugation of ligands allowing targeted delivery of the siRNA directly to tumor tissue. We will explore this in future experiments. Using the nanoparticle, we will also examine whether combining CHK1-Gemcitabine siRNA with a second siRNA (e.g. against WEE1) can further potentiate the therapeutic effect observed in vivo or demonstrate activity against other tumor types.

Based on these in vitro results, future studies will examine whether these gemcitabine containing siRNAs delivered IV in a nanoparticle or conjugated with ligands (to target specific cancer types), can also produce efficacy when used against tumors of lung cancer, ovarian cancer or TNBC.

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Ethical Guidelines: All xenograft animal studies were performed at Crown Bio in the US. The experiments were approved by the IACUC and performed under the conditions specified in the protocol.

Conflict of Interest: The authors declare no conflicts of interest

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