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Polymeric Nanogel-based Treatment Regimen for Enhanced Efficacy and Sequential Administration of Synergistic Drug Combination in Pancreatic Cancer

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Abbreviations: BLI- Bioluminescence Imaging; CDDP-Cisplatin; DMEM- Dulbecco's Modified Eagle's medium; Gem-Gemcitabine; MTT- (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); NG-Nanogel; PDAC-Pancreatic ductal adenocarcinoma; PEG: Polyethylene glycol; PMA: Poly(methacrylic acid); Pt-Platinum; STn-Sialyl Tn.

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers. A combination of Cisplatin (CDDP) and Gemcitabine (Gem) treatment has shown favorable clinical results for metastatic disease; both are limited by toxicities and non-targeted delivery. More than 80% of the PDAC aberrantly expresses Sialyl Tn (STn) antigen due to the loss of function of COSMC (Core 1 beta3Gal-T Specific Molecular Chaperone), a specific chaperone for the activity of core 1 β3-galactosyltransferase or C1GalT. Here, we report the development of polymeric nanogels (NGs) loaded with CDDP and coated with an anti-STn antigen specific antibody (TKH2 mAb) for the targeted treatment of PDAC. TKH2-functionalized, CDDP-loaded NGs delivered significantly higher amount of platinum into the cells and tumors that are expressing STn antigens. We also confirmed that a synergistic cytotoxic effect of sequential exposure of pancreatic cancer cells to Gem followed by CDDP can be mimicked by co-delivery of CDDP-loaded NGs (NG/CDDP) and free Gem. In a murine orthotopic model of PDAC, combined simultaneous treatment with Gem and targeted NG/CDDP significantly attenuated the tumor growth with no detectable acute toxicity. Altogether, these results suggest that combination therapy consisting of Gem followed by TKH2-conjugated CDDP NGs induces a highly synergistic therapeutic efficacy against pancreatic cancer. Our results offer the basis for development of combination drug regimens using targeted nanomedicines to increase treatment effectiveness and improve outcomes of therapy of PDAC.

Key words

TKH2 mAb, Cisplatin, Gemcitabine, Nanogels, Combination therapy, Pancreatic Cancer

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies, due to aggressive tumorigenicity, early metastasis and development of drug resistance to standard care chemotherapy. PDAC is projected to become the second-leading cause of death within the next decade with five-year survival rate about 6 % (Rahib et al., 2014). Since its approval in 1997, Gemcitabine (Gem) is considered as front-line treatment for advanced disease. However, the treatment options are limited after Gem failure. The combination of Gem with Cisplatin (CDDP) has been explored in clinical trials for metastatic disease. As a part of chemotherapeutic combination FOLFIRINOX (Folinic acid, 5-Fluorouracil, Irinotecan and Oxaliplatin), platinum compounds showed significant efficacy (Conroy et al., 2011). Cisplatin acts by damaging the DNA. It is known to first get converted into the aqua form within the cell by the replacement of the labile chloro groups with water molecules. This active form is then able to form covalently linked adducts with the DNA. This initial assault further activates a series of signaling pathways that ultimately lead to cell cycle arrest and apoptosis (Eastman, 1990). However, how exactly the apoptotic pathways are activated as a result of CDDP insult remain unclear. On the other hand, Gem is a deoxycytidine analog whose mechanism of activation involves conversion into phosphorylated active form followed by incorporation into the DNA as a false nucleotide (Hertel et al., 1988; Van Moorsel et al., 1999). Usually, another deoxynucleotide is added into the DNA before the synthesis stops. Another minor mechanism of action of Gem is its ability to inhibit ribonucleotide reductase, a key enzyme in the repair mechanism of the DNA. Many studies have reported the benefit of administration of Gem prior to that of cisplatin; with the reason of increased formation of

Pt-DNA adducts when the DNA had already been damaged and exposed due to the incorporation of deoxycytidine or active Gem (Van Moorsel et al., 1999). The Gem in turn inhibits the repair of the formed Pt-DNA adducts as well as reduces the efficacy of nucleotide excision repair by its ability to prevent the action of ribonucleotide reductase (Yang et al., 1995; McMahon et al., 2010). Conversely, when Pt compounds are administered prior to Gem, the formed Pt-DNA adducts can no longer allow for the incorporation of Gem and that leaves no scope for Gem to act. In this study, we show that Gem administration followed by CDDP exhibited a schedule-dependent synergistic cytotoxic activity in PDAC. However, this combination proved to be only marginally effective in actual practice due to combined increased toxicity of both the agents. We have shown that encapsulation of CDDP in polymeric NGs with cross-linked ionic cores enhanced its tumor accumulation and improved its safety profile. Further, sustained release profile of CDDP from NGs allows for the administration of free Gem and CDDP-loaded NGs in a single injection while still retaining schedule-dependent synergy of the combination.

Monoclonal antibodies are being used in targeted therapy to deliver chemotherapeutic agents directly to cancer cells. Interestingly, overexpression of tumor-specific carbohydrate antigen STn, was observed in many epithelial cancers and the highest frequency was detected in PDAC. Increased expression of STn antigen is correlates with unfavorable prognosis and shorter overall survival of patients. Previously, we have demonstrated that aberrant expression of these immature truncated O-glycans promote malignant features in cancer cells such as increased migration, invasiveness, basement membrane disruption and loss of cellular architecture (Radhakrishnan et al.,

2014). Since, STn has not been detected in healthy tissues (Ferreira et al., 2013; Loureiro et al., 2015) and considering its clinical relevance in human malignancies and available therapies based on this antigen, STn can be used as an important target in pancreatic cancer. Therefore, the current study was designed considering STn antigen as target to deliver CDDP in order to avoid off-target accumulation of this chemotherapeutic agent and explore its activity in combination with Gem.

Materials and methods

Reagents. Poly(ethylene glycol)-*b*-poly(metharcrylic acid) di-block copolymer, PEG_{170}-*b*-PMA_{180}, Đ = 1.45, was acquired from Polymer Source (Canada). Maleimide-PEG-amine (Mal-PEG-NH_2, 7.5 kDa) was obtained from JenKem Technology, USA. Gemcitabine hydrochloride, CDDP, 2-iminothiolane hydrochloride, ethylenediaminetetraacetic acid (EDTA), and all other chemicals were from Sigma-Aldrich (St Louis, MO). Cell culture media (RPMI-1640, DMEM) and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). MTT reagent was purchased from Research Products International (Prospect, IL).

Preparation of Nanogels (NGs). Polymeric nanogels were synthesized as described previously (Kim et al., 2009). In brief, PEG_{170}-*b*-PMA_{180} chains were assembled into PEG_{170}-*b*-PMA_{180}/ Ca^{2+} complexes ($[Ca^{2+}]/[COO^-]$ = 1.3 mol/mol) followed by cross-linking of the polyion chains with 1,2-ethylenediamine using 1-(3 dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) chemistry ([COOH]/[EDC] = 5; $[EDC]/[NH_2]$ = 1). The resulting NGs were purified by dialysis against water in the presence of chelator (100 mM EDTA) and ammonia (0.5%).

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CDDP loading and antibody conjugation to NGs. CDDP loading was achieved as previously described (Oberoi et al., 2011). Briefly, CDDP and NGs were dissolved in water ([CDDP]=1mg/mL, [CDDP]/[COO^-] = 0.5) and reacted for 48h at pH 9 and 37^{o}C. CDDP-incorporated NGs were concentrated and washed with water separated using and Amicon Ultra-15 centrifugal filter unit (MWCO 30 kDa). Thiolation of antibody (TKH2 or non-specific IgG) was performed in phosphate buffer (pH 8, 10 mM EDTA) using 2-iminothiolane (15 eq., 1 h) (Manjappa et al., 2011; Steinhauser et al., 2006). Thiolated antibody was purified using Zeba™ Spin desalting column previously equilibrated with phosphate buffer, pH 7, 10 mM EDTA as per manufacturer's protocol. Mal-PEG-NH_2 (10 eq) was then added to thiolated antibody and allowed to react for 2h. The excess of unreacted PEG was removed by ultrafiltration (MWCO 30 kDa). The PEGylated antibody was then coupled to NGs via amidation reaction with free carboxyl groups of in the presence of EDC ([COO-]/[PEG-NH_2] = 1.5). The mixture was passed through a Sepharose CL-6B column to remove unbound antibody (Nukolova et al., 2011). Resulting mixture contained both unmodified and antibody-conjugated NGs. Protein content of this mixture was determined by microBCA assay kit (Thermo Scientific) using BSA as standard as per the manufacturer's protocol.

Characterization of NGs. The size (hydrodynamic diameter, D_{eff}), polydispersity index (PDI), and zeta-potential of NGs were determined by dynamic light scattering (DLS). The measurements were performed in the automatic mode at 25^{o}C and analyzed using software provided by the manufacturer (Zetasizer, Malvern Instruments Ltd.). The reported values were based on the average of three separate measurements.

Cell culture conditions and Transductions. T3M4 wildtype (WT, either low or no expression of STn antigen) and T3M4 COSMC KO (SimpleCells, SC, aberrantly express STn antigens) cells were maintained as previously reported (Radhakrishnan et al., 2014). The cells were transduced with firefly luciferase and GFP expressing lentiviral vectors (F-Luc-GFP lentiviral vectors) as per manufacturer's instructions (Capital Biosciences, Rockville, MD) to obtain T3M4/Luc cell line.

NGs and CDDP cellular uptake. Cells (10^6 cells/well) were seeded in 24-well plates in DMEM 24 h prior exposure to Cy3-labeled TKH2 - NG or IgG-NG (0.5mg polymer/mL) for up to 2 h at 37°C. Subsequently, cells were washed three times in PBS, harvested with trypsin, pelleted (1500 rpm, 5 min), and re-suspended in PBS . The fluorescence of treated cells was analyzed using Becton Dickinson FACStar Plus based on 5000 events acquired for each sample and gated to exclude debris and dead cells. To assess CDDP cellular uptake, T3M4 WT and SC cells (100,000 cells/well) were plated in a 6-well plate and incubated for 2 days followed by treatment with TKH 2 – NG/CDDP or IgG – NG/CDDP for 1 h or 2 h . The drug concentration during the exposure was 161.5 μM in CDDP equivalents. After incubation, cells were washed in PBS, trypsinized, pelleted by centrifugation, and digested with 0.3 mL of concentrated nitric acid. Platinum concentrations was determined by inductively coupled plasma mass spectrometry (ICP-MS).

***In vitro* cytotoxic activity**. T3M4 WT and SC cells were plated at a 5000 cells/well density in 96-well plates and incubated for 24 h. Cells were then exposed to either free Gem or free CDDP or Gem + CDDP or Gem, 24 h + CDDP, 24 h or CDDP, 24h + Gem, 24 h or CDDP-NG or Gem + TKH2 – NG/CDDP or Gem + IgG – NG/CDDP at equivalent

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doses of CDDP (0 – 10 µg/mL or Gem 0 – 10 ng/mL) for total of 48 h in DMEM at 37ºC. CDDP: Gem molar ratio was about 1140:1 or 1000:1 w/w. Sterile Gem, CDDP and CDDP-NG solutions were prepared in dextrose (5%) and required dilutions were made in DMEM. After incubation, each well was washed with PBS and allowed to grow in fresh DMEM for 24 h. Cell viability was then analyzed using MTT assay (Mosmann, 1983) and IC_{50} values of different treatments were calculated using GraphPad Prism software. Synergy, antagonism or additive effects of drug combination were assessed by combination index (CI) analysis using CompuSyn software based on Chou-Talalay's method (Chou, 2010).

***In vivo* orthotopic pancreas tumor studies**. All animal experiments were performed with protocols approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. For this study, female nude mice (athymic nu-nu, 4-week old, Charles River Laboratories) were used. Animals were kept in AAALAC accredited facility and were quarantined for 7 days before the start of studies. To establish *in vivo* orthotopic pancreas tumor model T3M4/Luc cells (~2.5×10^5 cells) were injected directly into the pancreas. At day 14 after cell injection animals were randomly distributed into six groups (n = 10) and then treated with 5% dextrose (control group) or IgG – NG/CDDP or TKH2 – NG/CDDP or free Gem or combination of Gem + IgG – NG/CDDP or Gem + TKH2 – NG/CDDP at an equivalent dose of 4 mg/kg CDDP, or 20 mg/kg Gem. The formulations were administered via tail vain using q4d × 4 regimen. Sterile Gem solutions were prepared in dextrose (5%), and used alone or in admixture with corresponding CDDP or NG/CDDP solutions for drug combination treatment such that the final dose volume would be 100 µL per dose. Tumor growth (Bioluminescence imaging, BLI) and animal body weight were recorded every fourth day. Prior to imaging, mice were injected

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intraperitoneally with D-luciferin solution in sterile PBS (150 mg/kg), anaesthetized with isoflurane and then images were taken using IVIS-200 (Xenogen Corporation, Alameda, CA) as reported previously (Chai et al., 2013). Total bioluminescence signal in the regions of interest drawn around the whole abdomen region was quantified IVIS software and expressed as photons/s/cm^2/sr. The animals were sacrificed on day 15 of the commencement of the treatment. Organs and whole blood were collected for analysis and peritoneal cavities were checked for the presence of metastasis.

Sample preparation and Pt content measurement in tissues. ICP-MS analysis was used to quantify total Pt content in excised tissues (tumor, kidney, spleen, liver and lung, 3 mice per group). Thawed tissues samples of known weight were spiked with iridium internal standard and decomposed by wet-ashing with concentrated nitric acid (6 vol. equivalents) at 65°C at constant stirring overnight. Calibration range for the assay was 2-100 ng Pt/mL and necessary dilutions were made when the Pt concentration exceeded this range. Assay sensitivity was 0.8 ng of Pt/mL with variability not exceeding 5%.

Blood chemistry and histopathology. Following animal sacrifice blood was collected in heparin tubes. The levels of markers for hepatic and renal functions were determined using Vetscan VS (Abaxis). Excised tissue samples were fixed in 10% neutral buffered formalin and then processed for paraffin embedding, preparation of 5 micron thick sections, and stained with hematoxylin and eosin (UNMC Tissue Sciences Facility).

Statistical analysis. Statistical comparisons for *in vitro* studies were done using Student's t-test. One-way analysis of variance was utilized for the analysis of the mean BL signal intensity and body weight data. Differences in tumor metastasis between groups were analyzed using two-tailed Fisher's exact test. A statistically significant difference

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was considered at $p < 0.05$. Statistical analysis was performed using GraphPad Prism 5 software.

Results

Schedule-dependent cytotoxicity of Gem - CDDP combination. The combination was found to be synergistic only when the T3M4 SC cells were first exposed to Gem followed by CDDP, with the CI value for this regimen being <1 (CI = 0.17). When CDDP administration was followed by Gem, the effect of the combination was antagonistic as the CI value exceeded 1 (CI = 1.98) while co-administration of Gem and CDDP provided a mere additive effect with CI ~1 (**Table 1**). These results indicate that a formulation approach involving immediate availability for Gem while delayed availability for CDDP would help in retaining the schedule-dependent synergy of action of Gem – CDDP combination. To achieve this, CDDP was formulated in NGs with a sustained release profile with a targeting antibody conjugated on the surface of NGs to improve delivery of CDDP to tumor tissue. Gem would be administered as a free drug, making it available immediately for uptake upon intravenous administration.

Preparation and characterization of CDDP-loaded, antibody-conjugated NGs. NGs were produced by 1) self-assembly of ionic block copolymer PEG_{170}-*b*-PMA_{180} in the presence of $CaCl_2$ followed by 2) crosslinking the PMA chains and 3) removal of the Ca^{2+} ions (Kim et al., 2009). The DLS analysis revealed the formation of NGs with hydrodynamic diameters of about 114 nm (ζ- potential = −25 mV) and narrow size distribution (PDI<0,1). Loading of CDDP (~25 % w/w) occurs through coordination interactions of this drug with the COOH functionalities of NGs and lead to a reduction in

size (D_{eff} = 93 nm) and increase in ζ-potential to -14 mV. For conjugation, antibodies (either TKH2 or IgG) were first thiolated using Traut's reagent and PEGylated using maleimide-PEG-NH_2. We utilize longer PEG chains (M_w = 7.5 kDa) as spacers between antibody and NG to avoid steric effects of PEG corona of NGs that can influence ligand-receptor binding. The terminal amino group of PEG spacer was then used to conjugate the antibody to free carboxylate groups in the cores of CDDP-loaded NGs. Such conjugation strategy is non-specific for the site as well as number of PEG spacers conjugated per antibody molecule and leads to a mixture of NGs carrying varying number of antibody molecules per NG as well as non-modified NGs. Subsequently, free antibody were separated by size exclusion chromatography using a Sepharose CL-6B column and the obtained mixture of antibody conjugated NGs was used in further experiments. Protein content was ~65 µg/mg polymer for both antibodies, as determined by microBCA assay. Modification of the CDDP/NGs surface with either TKH2 or IgG led to an increase of the particle size (from 93 nm to 135 nm), while the PDI values still remained relatively low (**Table 2**). The ζ-potential of the antibody-conjugated NGs was marginally increased compared to that for the non-targeted NGs (**Table 2**). The observed trend can be attributed to shielding effect of additional PEG chains tethered to NG surface as well as a decrease of the number of charged PMA carboxylate groups due to reaction with the PEG spacers.

Cellular Interactions of antibody-conjugated NGs. In order to analyze the binding affinity of the targeting antibody, either TKH2 or IgG were conjugated to FITC-labeled NGs as described above and the cellular association of the NGs was estimated using flow cytometry. Both T3M4 SC (STn positive) and T3M4 WT (STn negative) cells were

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treated with FITC-labeled TKH2 - NG or IgG - NG. As shown in **Figure 1A** and Supplemental **Figure S1**, the uptake of TKH2 - NG is significantly higher ($p < 0.01$) than IgG - NG in T3M4 SC cells while the uptake for both types of NGs remains the same in T3M4 WT cells (**Figure 1B**). Also, the percentage of parent gated cells positive for TKH2-NGs is significantly increased in T3M4 SC cells as compared to T3M4 WT cells in time-dependent manner ($p<0.05$) (**Figure 1C**). Also, we measured the Pt content by ICP-MS in T3M4 cells that were treated with either CDDP-loaded TKH2-NG or CDDP-loaded IgG -NG. The Pt content was significantly higher in T3M4 SC cells treated with TKH2 - NG as compared to IgG - NG ($p<0.05$), while both the treatments showed similar Pt levels in case of T3M4 WT cells (**Figure 1D**).

***In vitro* cytotoxic activity of CDDP-loaded, antibody-conjugated NGs**. The isogenic PDAC cells (T3M4 WT and SC) were treated with various combinations of free Gem and CDDP-loaded NGs, which were decorated with either the targeting anti-STn mAb TKH2 or IgG as the non-targeted control. Combined treatments of Gem and CDDP formulations were substantially more effective in these cells than CDDP alone or NG/CDDP. The cytotoxic dose of CDDP in NGs was almost 40-fold lower when used in combination with Gem (**Table 3**). As seen with many targeted delivery systems (Desale et al., 2015), the uptake of targeted and non-targeted NGs can become practically the same over extended period of time, given the stagnant environment of an *in vitro* assay. The same effect is expected to be the cause for similar IC_{50} values determined for cells treated with targeted (TKH2 – CDDP/NG) or non-targeted NGs (IgG – CDDP/NG). This trend was also seen across the STn negative T3M4 WT cells. Notably, the synergistic cytotoxic interaction

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between Gem and CDDP when delivered to cells sequentially (Gem, 24h + CDDP, 24h, Table 3) was retained upon simultaneous treatment with Gem and CDDP/NGs.

Antitumor activity in orthotopic pancreas tumor model. The therapeutic potential of drug combination was tested in an orthotopic pancreatic cancer mouse model. Mice were injected with T3M4 SC/Luc cells (2.5×10^5 cells/30 µL) directly into the pancreas. Tumors were detectable from day 9 by BLI and treatment was started from day 14 of post-implantation of tumor cells. Tumor bearing animals were treated via the tail vein following q4d × 4 regimen at equivalent doses of 4 mg/kg CDDP and 20 mg/kg Gem. The tumor progression for each individual treatment is shown in **Figure 2A**. Monotherapy with either TKH2 - NG/CDDP (targeted formulation) or Gem significantly slowed down the growth of the primary tumor ($p < 0.05$). Treatment with Gem + TKH2 - NG/CDDP combination further retarded the tumor growth as compared to Gem alone ($p < 0.05$) or TKH2 - NG/CDDP ($p < 0.01$). Combination of Gem with IgG - NG/CDDP (non-targeted formulation) also led to suppression of tumor growth compared to IgG - NG/CDDP alone ($p < 0.01$) but did not exhibit better treatment efficacy compared to Gem monotherapy. Treatment with Gem + TKH2 - NG/CDDP targeted regimen displayed more pronounced tumor-suppressive effects than Gem + IgG - NG/CDDP, however the difference did not achieve statistical significance (**Figure 2B**). Further, to analyze the anti-metastatic potential of combination treatment, all major organs were dissected and observed for visible metastasis. Notably, peritoneal metastases were not observed in any of the animals ($n = 10$) that received combination of both Gem and CDDP/NG, either targeted or non-targeted (Supplemental **Figure 2**). In most organs, the combination treatment showed better antimetastatic effect than monotherapies.

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Pt content in tumor and other organs. To compare the effectiveness of STn antigen-targeted drug carriers in delivering CDDP to tumors, tissues from 3 animals that were randomly selected from each treatment group were digested and tested for Pt content using ICP-MS. It was found that Pt contents in tumors of animals treated with TKH2 - NG/CDDP were significantly higher than those treated with IgG - NG/CDDP ($p < 0.01$) irrespective of the presence of Gem (**Figure 3**). An interesting observation is that the presence of Gem itself helped in enhancing the delivery of CDDP to the tumor tissue: higher Pt levels were detected in tumors of animals treated with Gem + TKH2 - NG/CDDP vs TKH2 - NG/CDDP ($p < 0.05$). Higher levels of Pt accumulation were also observed in liver and spleen, organs of the mononuclear phagocyte system, but the effects of targeted TKH2-mediated delivery as well as overall enhanced delivery of CDDP due to the presence of Gem were observed exclusively in the tumor tissue and not in other healthy organs.

Clinical chemistry parameters and toxicity profiles. During the course of the treatment, body weights of animals were routinely monitored, and no significant changes were observed across different treatment groups (Supplemental **Figure 3**). Blood collected from animals sacrificed at 72 h post final dosing was subjected to analysis of clinical chemistry parameters indicative of renal and hepatotoxicity. As shown in **Table 4**, average alkaline phosphatase (ALP), alanine aminotransferase (ALT), and blood urea nitrogen (BUN) values were found to be in the normal range across all treatment groups, which indicated that no short-term toxicity occurred to the animals as a result of the treatment. Furthermore, no histopathological changes in healthy organs were seen in any

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of the animals, providing additional proof that all treatments were well-tolerated by the animals and no short-term toxicity was evident (Supplemental **Figure 4**).

Discussion

PDAC remains a major health burden owing to its obstinate nature to therapeutic agents that impose poor prognosis of the disease. For the last two decades, Gem has become the reliable drug regimen for advanced PC as it showed significant betterment in the median overall survival of patients in clinical trials (Goess and Friess, 2018; Sinn et al., 2017). Huge bodies of research evidences suggest the advantages of using combinatorial therapy of Gem with other cytotoxic agents (Chen et al., 2018; Chakraborty et al., 2018; Hamada et al., 2017; Von Hoff et al., 2013). Though these combination therapies show significant improvement in prolongation of overall median survival in clinical trials, long-term survival of patients remains poor (ACS, 2017), demanding the need of novel effective therapeutic approaches against the disease progression. Targeted delivery of precision medicine combined with chemotherapeutic drugs is believed to improve the therapeutic effects. To address this, we have developed CDDP encapsulated polymeric NGs conjugated with STn antigen-specific TKH2 monoclonal antibody, which allows targeted delivery of CDDP to tumors.

In our preliminary *in vitro* studies, we found that administration of Gem followed by CDDP is highly effective against STn antigen expressing PDAC cells (T3M4 SC) (Table 1). We have observed that immediate availability of Gem with delayed availability of CDDP shows a strong synergy of cytotoxic effect on T3M4 SC cells. When the drugs were applied in the reverse sequence, that is CDDP followed by Gem, the effect was found to

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be antagonistic, while simultaneous administration of the two agents showed a mere additive effect. Our data are in the agreement with the previously reported synergism between Gem and platinum agents in different cancer cell lines (Wang et al., 2010; Duangjai et al., 2014; Van Moorsel et al., 1999; Bergman et al., 1996; Huang et al., 1991) when the cells were pretreated with Gem followed by platinum agents. Overall, these results highlighted the importance of sequence-specific delivery of two drugs when used in combination to achieve maximal cytotoxic effect. We have previously developed a novel platform for drug delivery based on nanoscale size polymeric NGs. The NGs are prepared through a self-assembly process, which involves formation of a cross-linked polyion core micelle coated by inert PEG chains (Bronich et al., 2005). The resulting NGs are water-swollen, have very low buoyant density and can encapsulate large amounts (~30 % w/w) of water-soluble drugs through electrostatic or coordination interactions of these drugs with COOH functionalities (Kim et al., 2009; Oberoi et al., 2011). We have previously demonstrated the beneficial effects of CDDP loading into the NGs as a strategy to improve PK, antitumor efficacy and reduce CDDP-associated nephrotoxicity in ovarian cancer model (Oberoi et al., 2012). Moreover, the surface of NGs was decorated with various targeting ligands (small molecules, polypeptides and antibodies) and successful delivery of targeted NGs to tumors has been confirmed (Nukolova et al., 2011; Nukolova et al., 2013).

STn antigen is an attractive target as its expression in normal adult tissue is rare and is largely restricted to cancer cells (Julien et al., 2012; Munkley, 2016). In this context, monoclonal antibody TKH2 which is known to bind STn antigen with high specificity, is an excellent candidate ligand in order to facilitate the delivery of CDDP to the pancreatic

cancer cells. Therein, model NGs based on PEG-*b*-PMA copolymer were loaded with CDDP and then modified with TKH2 antibody using the bi-functional PEG-linker. IgG-linked CDDP/NGs were used as non-targeted control. The resultant NGs were about 135 nm in diameter, displayed overall negative net charge and were able to efficiently incorporate CDDP (loading ~25% wt). Importantly, despite the nonspecific method of antibody conjugation to NGs that may hinder binding sites of antibody, the binding affinity of TKH2 to its target receptor was retained, which was demonstrated by a significantly higher cellular association of TKH2-NGs with cancer cell that are known to overexpress STn antigen as compared to control IgG-NGs. In contrast, the uptake of both specific as well as non-specific antibody conjugated NGs was similar in T3M4 WT cells (**Figure 1**) indicating the target specificity of TKH2 antibody against STn antigen. However, despite the enhanced cellular association of TKH2-NGs, the combination of Gem + TKH2-CDDP/NG had a lower potency as compared to the free drug combination delivered to cells sequentially (**Table 3**). This is likely due to several factors including (1) lower accumulation and vesicular sequestration of CDDP/NGs in the cells compared to free drug treatment and (2) slow release of Pt species from a nanocarrier that can lead to reduced cytosolic drug pool. Indeed, we have previously shown that rate of release of Pt species from CDDP-loaded NGs is slow with about 15% of Pt species released during 48 h (Oberoi et al., 2011).

Combined Gem + TKH2-CDDP/NG therapy was further evaluated in mouse orthotropic pancreatic cancer model. This model is known to closely mimic the tumorigenesis within the human body (Takahashi et al., 2011) and provide the ability to study site-specific dependence of therapy. To this end, T3M4 SC/Luc cells were

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inoculated directly into the pancreas and disease progression was monitored by noninvasive *in vivo* bioluminescence imaging. It was found that the treatment with combination of Gem and targeted TKH2 - NG/CDDP significantly slowed down the growth of the primary pancreatic tumor (**Figure 2**). This observation correlates well with the results obtained in *in vitro* cytotoxicity assays, wherein sequential administration of free Gem followed by free CDDP showed maximum synergy of cytotoxic activity (**Table 1**). *In vivo*, this sequential delivery is likely to be achieved by free Gem being immediately available to the tumor cells and therefore presented first followed by CDDP that is slowly released from the NGs. Furthermore, the presence of TKH2 antibody on the surface of NGs greatly enhanced the association of the drug carrier with STn antigen-positive tumor cells (**Figure 3**). Such selective binding and/or uptake of TKH2 - NG/CDDP by cancer cells could result in longer retention of nanocarriers within the tumor, avoid their quick re-entering into blood circulation and, thus, offer some advantages over passively targeted formulations (Baklaushev et al., 2015). Indeed, analysis of Pt content in tumors collected post 72 h of the last injection revealed that inclusion of targeted NGs into treatment regimens led to a significantly higher tumor accumulation of Pt species compared to non-targeted NGs ($p < 0.01$). Notably, TKH2-NG/CDDP displayed significantly higher levels of Pt in tumors when were applied in combination with Gem. Although the mechanisms underlying this phenomenon are not clear at the moment, recent studies in orthotopic pancreatic tumor xenografts showed an increased accumulation of macrophages in tumor tissue of Gem-treated mice (Deshmukh et al., 2018). Thus, an observed elevated accumulation of Pt in tumor tissues might be facilitated through uptake of NG/CDDP by tumor-associated macrophages. Future studies will need to confirm this possibility and

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assess the functional consequences of our observation. The abnormal characteristics of tumor microenvironment such as uneven vascular distribution, heterogeneous blood flow, elevated interstitial fluid pressure, tumor stromal compartment contribute to the transport barriers and compromise the ability of nanocarriers with sizes above 50 nm to penetrate through the tumor (Jain, 1990). This is especially true for poorly vascularized cancers like PDAC (Cabral et al., 2011), and, therefore, the development of NG-based carriers with size below 50 nm may further improve the delivery of platinum agents into the tumor, Furthermore, the introduction of TKH2 antibodies to NGs can also affect the PK, biodistribution, and tumor uptake of nanocarriers. Additional pharmacokinetic studies will be necessary to investigate the association between distribution of the targeted NGs at the tumor and the whole body and their therapeutic effect and allow to optimize the treatment schedule of drug combination.

Overall, our proof-of-concept studies demonstrated that TKH2 decorated NGs were effective in delivering higher payloads of CDDP to the tumor site. This lead to a better therapeutic efficacy of combined GEM + TKH2-NG/CDDP in terms of tumor growth retardation compare to other treatments. Collectively, these data confirm the potential of using Gem in combination with platinum-based cytotoxic agents like CDDP, the effectiveness of which can be further enhanced by using targeted delivery approaches for PDAC treatment.

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Authorship Contributions

Participated in research design: Hollingsworth, Radhakrishnan, and Bronich.

Conducted experiments: Soni, Caffrey, Mehla, Connell, and Radhakrishnan.

Performed data analysis: Soni, Lei, Lele, Thomas, Sagar, Radhakrishnan, and Bronich.

Wrote or contributed to the writing of the manuscript: Soni, Thomas, Radhakrishnan, and Bronich.

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Footnote:

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Figure Legends

Figure 1. Cellular association of FITC-labeled TKH2 – NG and IgG – NG with (**A**) T3M4 SC cells and (**B**) T3M4 WT cells. (**C**) Percentage of parent gated cells as a function of time. Cells were treated with FITC-labeled TKH2-NGs at 0.5 mg/mL polymer. Protein content was 65 μg/mg polymer. *p < 0.05. Data are mean ± SD (n = 3). (**D**) Whole-cell Pt accumulation in T3M4 SC and T3M4 WT following incubation with TKH2 – NG/CDDP or IgG – NG/CDDP cells (CDDP equivalent concentrations of 161.5 μM, 37 °C) as measured by ICP-MS (n=3).

Figure 2. *In vivo* anti-tumor efficacy of Gem +TKH2-NG/CDDP regimens in T3M4 SC/ Luc orthotopic pancreatic cancer xenograft-bearing female nude mice. (**A**). Bioluminescence images of each 3 mice representative for 10 mice per experimental arm after treatment (day 15). (**B**). Comparison of tumor growth inhibition following IV administration of 5% dextrose (control); IgG-NG/CDDP; TKH2-NG/CDDP; Gem alone; Gem + IgG-NG/CDDP, and Gem + TKH2-NG/CDDP. Drug combinations were injected in 100μl volume every 4th day for a total of 4 injections at 4 mg/kg CDDP and 20 mg/kg Gem equivalent doses (indicated by arrows). Data presented in terms of relative bioluminescence units (R_t/R_0) to the day when treatment was initiated for individual animal. Values indicated are mean ± SD (n=10). *p < 0.05, **p < 0.01.

Figure 3. Tissue distribution of platinum in different treatment groups as determined by ICP- MS. Mice were sacrificed at day 15 of the treatment with TKH2 – NG/CDDP or Gem + TKH2 – NG/CDDP or IgG – NG/CDDP or Gem + IgG – NG/CDDP. Data are represented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

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Table 1. Comparison of IC_{50} values for different sequences of administration of Gemcitabine and CDDP against T3M4 SC cells as determined by the MTT assay.

Treatment schedule [a]	**IC_{50} (µg/ml)**[b]
CDDP, 24h → Gem, 24h	1.69 ± 0.052 (CI = 1.98)
Gem, 24h → CDDP, 24h	0.028 ± 0.004 (CI = 0.17)
CDDP-Gem co-administration	1.51 ± 0.15 (CI = 0.95)
CDDP	1.18 ± 0.14
Gem[c]	0.48 ± 0.03

[a] T3M4 SC cells were treated for total of 48 h.

[b] IC_{50} values were calculated with respect to CDDP and represent mean ± SD of at least three independent experiments. CI - combination index, calculated at IC_{50}.

[c] IC_{50} of Gem expressed in ng/mL.

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Table 2. Physicochemical characteristics of drug-loaded NGs

Sample (pH 7.4)	D_{eff} (nm)	PDI	Zeta-potential (mV)
NG	114 ± 2	0.09 ± 0.02	-25.0 ± 1.8
CDDP/NG	93 ± 1	0.10 ± 0.02	-14.0 ± 1.3
TKH2 – NG/CDDP	135 ± 2	0.15 ± 0.02	-9.6 ± 1.0
IgG – NG/CDDP	137 ± 1	0.14 ± 0.01	-10.7 ± 2.1

Effective diameter (D_{eff}), polydisperity index (PDI) and ζ-potential were determined in water (pH6.5).

Values represent mean ± SD (n=3).

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Table 3. IC_{50} doses for drugs and drug formulations against pancreatic cancer cell lines.[a]

Treatment schedule	IC_{50} (μg/mL)	
	T3M4 SC	**T3M4 WT**
Gem + TKH2 – NG/CDDP	0.130 ± 0.014	0.140 ± 0.003
Gem + IgG – NG/CDDP	0.150 ± 0.007	0.160 ± 0.006
Gem, 24h + CDDP, 24h[b]	0.030 ± 0.003	0.035 ± 0.002
Gem[c]	0.50 ± 0.03	0.80 ± 0.03
Free CDDP	1.22 ± 0.15	1.55 ± 0.04
NG/CDDP	6.45 ± 0.30	7.77 ± 0.23
Gem + CDDP co-administration	1.20 ± 0.08	1.49 ± 0.19

[a] T3M4 (WT and SC) cells were treated with Gem, CDDP, NG/CDDP, Gem + CDDP, Gem + TKH2-NG/CDDP and Gem + IgG-NG/CDDP for 48 hours and the cell viability was measured by MTT assay. IC_{50} values were calculated with respect to CDDP and represent mean ± SD of at least three independent experiments.

[b] The cells were treated with free Gem for 24 h followed by addition of CDDP and incubation for total 48 h.

[c] IC_{50} of Gem is expressed in ng/mL.

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Table 4. Clinical chemistry parameters as assessed by whole blood analysis

Treatment group	ALP (IU/L)	ALT (IU/L)	BUN (mg/dL)
Gem + TKH2 – NG/CDDP	14.5 ± 0.7	46	17
Gem + IgG – NG/CDDP	13 ± 2.65	50 ± 3	17.6 ± 1.53
TKH2 – NG/CDDP	15	39.5 ± 5.5	15 ± 1.15
IgG – NG/CDDP	15	38 ± 2.6	15 ± 0.6
Free Gem	15.5± 2.1	45.5 ± 3	15 ± 4
Control	14 ± 0.8	46.5 ± 3.5	17 ± 2.8

Clinical chemistry parameters as assessed by whole blood analysis from animals sacrificed on day 15. Data are represented as mean ± SD (n = 3).

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Figure 1

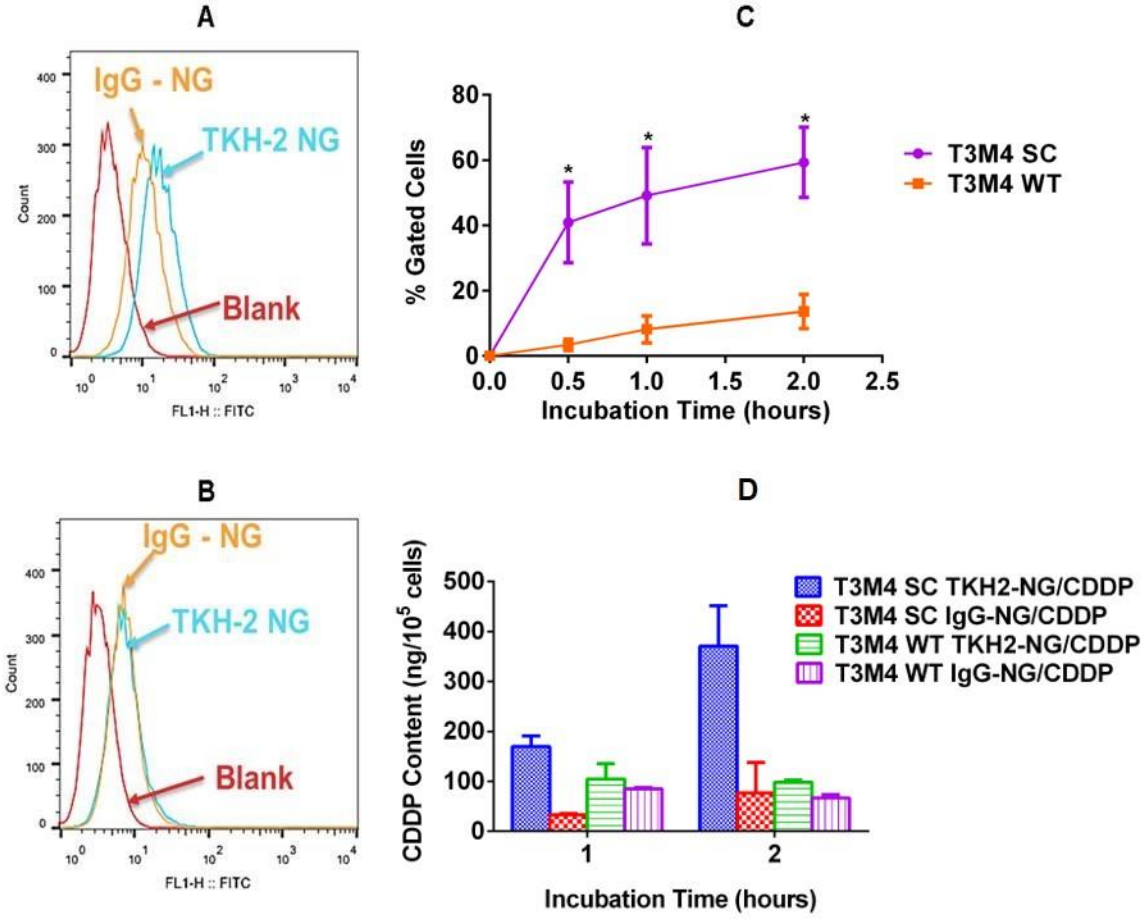

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Figure 2

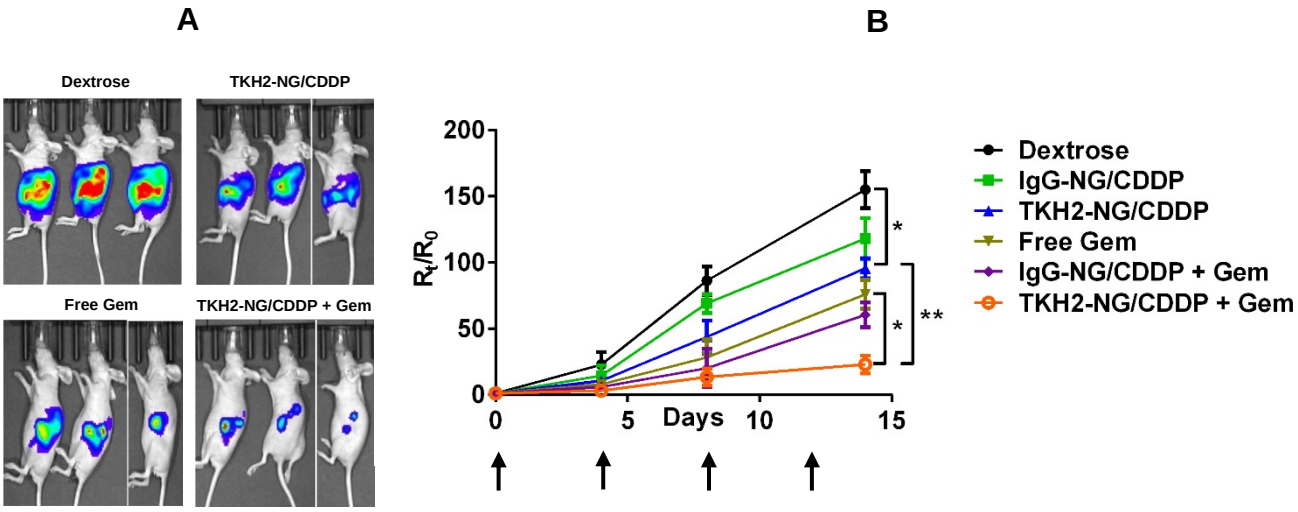

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Figure 3