PANCREATIC CANCER

A Preclinical Evaluation of Minnelide as a Therapeutic Agent Against Pancreatic Cancer

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Pancreatic cancer is one of the most lethal human malignancies with an all-stage 5-year survival frequency of <5%, which highlights the urgent need for more effective therapeutic strategies. We have previously shown that triptolide, a diterpenoid, is effective against pancreatic cancer cells in vitro as well as in vivo. However, triptolide is poorly soluble in water, limiting its clinical use. We therefore synthesized a water-soluble analog of triptolide, named Minnelide. The efficacy of Minnelide was tested both in vitro and in multiple independent yet complementary in vivo models of pancreatic cancer: an orthotopic model of pancreatic cancer using human pancreatic cancer cell lines in athymic nude mice, a xenograft model where human pancreatic tumors were transplanted into severe combined immunodeficient mice, and a spontaneous pancreatic cancer mouse model ($KRas^{G12D}$; $Trp53^{R172H}$; Pdx-1Cre). In these multiple complementary models of pancreatic cancer, Minnelide was highly effective in reducing pancreatic tumor growth and spread, and improving survival. Together, our results suggest that Minnelide shows promise as a potent chemotherapeutic agent against pancreatic cancer, and support the evaluation of Minnelide in clinical trials against this deadly disease.

INTRODUCTION

Despite recent advances in our understanding of cancer biology and the development of novel chemotherapeutic agents, a diagnosis of pancreatic cancer is associated with poor prognosis. In the United States alone, more than 40,000 persons are diagnosed with pancreatic cancer each year and about the same number succumb to this disease (*1*). The all-stage 5-year survival rate for this cancer is a disheartening 5%, and this rate has remained largely unchanged over the last 3 decades (*1*). Multiple factors contribute to this overall poor prognosis, including detection at a late stage, aggressive tumor biology, and a dearth of effective chemotherapeutic agents. Surgical removal is the only opportunity for a cure from the disease. However, only 10 to 20% of patients present early enough to be considered candidates for curative surgery (*2*). In contrast, 40% of patients have metastatic disease at presentation; these patients are mainly treated with palliative therapy (*2*). The remaining 40 to 50% of pancreatic cancer patients have locally advanced disease that is not amenable to surgical resection (*2*). The current chemotherapeutic agent of choice for pancreatic cancer is gemcitabine, which was approved by the U.S. Food and Drug Administration (FDA) in 1996: Gemcitabine confers a median survival advantage of only 6 months (*3*), an improvement of only 1 month over its predecessor [5-fluorouracil (5-FU)]. Addition of erlotinib, an epidermal growth factor receptor inhibitor recently approved by the FDA, adds only two more weeks to the average overall survival time (*4*). The dismal prognosis for most pancreatic cancer patients underscores an urgent need to discover and develop more effective therapeutic strategies against pancreatic cancer.

We and others have previously shown that triptolide (*5*), a diterpenoid triepoxide isolated from the medicinal vine *Tripterygium wilfordii* Hook F, is highly effective against a variety of cancer types, including pancreatic cancer (*5*), colon cancer (*5*), neuroblastoma (*6*), and cholangiocarcinoma (*7*). Although initially triptolide was considered to hold great promise as a chemotherapeutic agent (*5*), its potential clinical use is limited because it is poorly soluble in water but soluble in organic solvents.

To overcome issues with solubility, we have designed and synthesized a highly water-soluble analog of triptolide, named Minnelide. Here, we present the preclinical assessment of Minnelide in multiple cell line and tumor-derived animal models of pancreatic cancer. We compared the in vitro efficacy of Minnelide with that of its parent compound (triptolide). Subsequently, we evaluated the efficacy of Minnelide in multiple animal models of pancreatic cancer, including orthotopic models using established pancreatic cancer cell lines, a human xenograft model, and a genetic mouse model of pancreatic cancer. Each model has its benefits; thus, the combined results from testing of Minnelide in these complementary systems are expected to be more predictive of performance in clinical studies. We observed that Minnelide effectively decreased tumor burden, tumor-associated morbidity, and locoregional spread as well as increased overall survival in multiple animal models of pancreatic cancer. Minnelide was more effective than gemcitabine, the current standard of care for pancreatic cancer, in reducing tumor volume in an orthotopic model of pancreatic cancer. The results presented here pave the way for future clinical studies with Minnelide in patients with pancreatic cancer.

RESULTS

Minnelide is synthesized from triptolide and bioconverted back to the parent compound in the presence of phosphatase

Minnelide, a white hygroscopic powder, was synthesized using a previously reported procedure (*8*). The details of the synthesis and the full

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characterization of compounds are available in the patent application (WO/2010/129918). In brief, Minnelide was synthesized by reacting triptolide with acetic acid and acetic anhydride in dimethyl sulfoxide (DMSO) at room temperature for 5 days to generate an intermediate form as a white foam. Subsequent reaction with dibenzylphosphate and *N*-iodosuccinimide in dry methylene chloride generated a dibenzyl ester derivative. Reductive removal of the dibenzyl group with hydrogen gas on palladium on carbon generated the corresponding dihydrogen phosphate group; subsequent reaction of this dihydrogen phosphate with sodium carbonate produced 14-*O*-phosphonooxymethyltriptolide disodium salt (Minnelide) as a white powder (Fig. 1). The purity of Minnelide that we used in our investigations was >95% as determined by high-performance liquid chromatography (HPLC). The kinetics of the water-soluble Minnelide was evaluated by assessing its degradation into the parent compound triptolide in the presence of alkaline phosphatase. As shown in Fig. 1, phosphatases, which are present in all tissues in the body including the blood (*9*), were expected to cleave the phosphate ester group, thereby generating a chemically unstable *O*-hydroxymethyl intermediate that would spontaneously release formaldehyde and triptolide in vivo. To assess the kinetics of Minnelide in an in vitro system, we carried out the bioconversion of Minnelide into triptolide in the presence of alkaline phosphatase in glycine buffer (pH 9.8). Both the disappearance of Minnelide (Fig. 2A, left) and the formation of triptolide (Fig. 2A, right) were measured. A first-order degradation rate constant was calculated by fitting the concentration remaining versus incubation time. The degradation half-life ($t_{1/2}$) of Minnelide was determined to be 2 min, showing rapid conversion of the modified drug into its parent compound.

Minnelide decreases cell viability of pancreatic cancer cells in vitro

To assess the effect of Minnelide on cell viability in pancreatic cancer cell lines, we treated S2-013, MIA PaCa-2, S2-VP10, and Panc-1 cells with 0 or 200 nM Minnelide in the presence of serum-free medium with or without alkaline phosphatase at 37°C. These concentrations were based on previously used concentrations of triptolide that were effective in vitro (*5*). As expected, because the conversion of Minnelide to triptolide requires the activity of alkaline phosphatase, Minnelide is ineffective in the absence of phosphatase. All cell lines studied show significantly decreased cell viability 48 hours after Minnelide treatment in the presence, but not in the absence, of phosphatase. The decrease in cell viability induced by 200 nM of activated Minnelide (50% of control) was similar to that caused by the same concentration of the parent compound, triptolide (Fig. 2B). The twofold difference in response between MIA PaCa-2 and the S2 cell lines (S2-013 and S2-VP10) may be attributed to the origin of these lines: The former is derived from a primary tumor, whereas the latter two are derived from metastases.

Minnelide decreases tumor burden in an orthotopic pancreatic cancer model

We next proceeded to test the effect of Minnelide administration on pancreatic tumors in vivo using an orthotopic pancreatic cancer mouse model in which cells were implanted into the tail of the pancreas of athymic nude mice. Animals with MIA PaCa-2–derived human pancreatic tumors were randomized into different treatment groups, and intraperitoneal injections of either saline, triptolide [0.2 mg/kg QD (once a day)], or Minnelide [0.1 to 0.6 mg/kg QD or BID (twice a day)] were started 12 days after tumor cell injection (day 1 in Fig. 3A) ($n = 10$ in each group). No alkaline phosphatase treatment was required to activate Minnelide in vivo because this enzyme is abundantly present in blood and all other tissues in the body (*9*). Treatment was continued until day 60, and mice were subsequently maintained without treatment for another 30 days (*9*). In accordance with University of Minnesota animal protocols, animals were sacrificed at earlier time points if signs of morbidity were observed, as described in Materials and Methods. The experiment was terminated by sacrifice of surviving mice at day 90, and analyses of tumor weight and volume were performed. Minnelide treatment led to a marked decrease in tumor weight and volume at the end of treatment. The average tumor weight was 3291.3 ± 216.7 mg in control mice; at the most effective dose, tumor weight was 373.0 ± 142.6 mg in Minnelide-treated mice (0.15 mg/kg BID; $P = 0.0006$, control versus 0.15 mg/kg BID) and 653.0 ± 410.9 mg in triptolide-treated mice (0.2 mg/kg QD; $P = 0.001$, control versus 0.2 mg/kg QD) (Fig. 3C). The average tumor volume measured was 2927.06 ± 502.1 mm^3 in the control group and 245.0 ± 111.4 mm^3 or 473.0 ± 291.9 mm^3 in Minnelide (0.15 mg/kg BID)– and triptolide (0.2 mg/kg QD)–treated mice, respectively (Fig. 3B). Thus, both Minnelide and triptolide were able to significantly prevent tumor growth.

Kaplan-Meier survival curves revealed that all doses of Minnelide used resulted in nearly 100% survival (90 to 100%), whereas only 10% of control saline-injected mice were alive at the end of the experiment. All deaths in the control group were due to tumor burden, as assessed by detailed necropsy, whereas only one death (0.1 mg/kg group) in the treated animals was due to tumor burden. One death in the 0.2 mg/kg triptolide group and one death in the 0.3 mg/kg Minnelide group were not related to tumor burden (Fig. 3, A and D; necropsy showed no tumor). Whereas all mice (10 of 10) in the saline-injected control group formed large tumors, either no tumors or small tumors were visible in Minnelide-treated animals from the 0.1 and 0.3 mg/kg QD or 0.15 mg/kg BID groups [9 of 10 (0.1 mg/kg), 9 of 10 (0.3 mg/kg), and 6 of 10 (0.15 mg/kg)], suggesting that Minnelide treatment prevents tumor formation.

Although our study suggests that Minnelide is not toxic at the doses tested for 60 days in immunocompromised animals, toxicity studies were conducted on immunocompetent animals (C57BL/6) at doses of 0.3 and 0.6 mg/kg QD for 29 days. Minnelide at a dose of 0.3 mg/kg demonstrated no significant differences in alanine

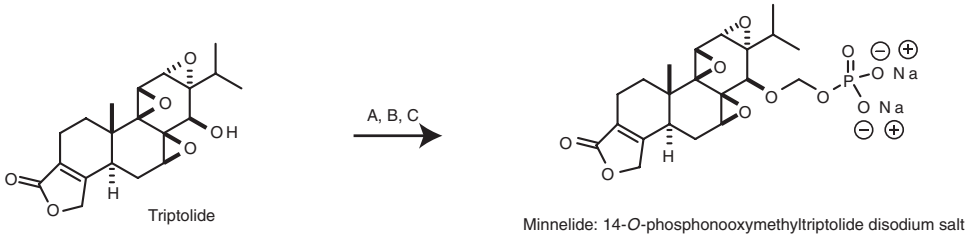


Fig. 1. Minnelide is synthesized from its parent compound, triptolide. Schematic of the synthesis of Minnelide from triptolide. (A) DMSO, Ac_2O, AcOH, 5 days, 52%. (B) Dibenzylphosphate, 4-Å molecular sieves, *N*-iodosuccinimide, dichloromethane, tetrahydrofuran, 5 hours, 80%. (C) (i) H_2, Pd/C, room temperature, 3 hours; (ii) $NaCO_3$, 90%; purity of Minnelide >95% (by HPLC).

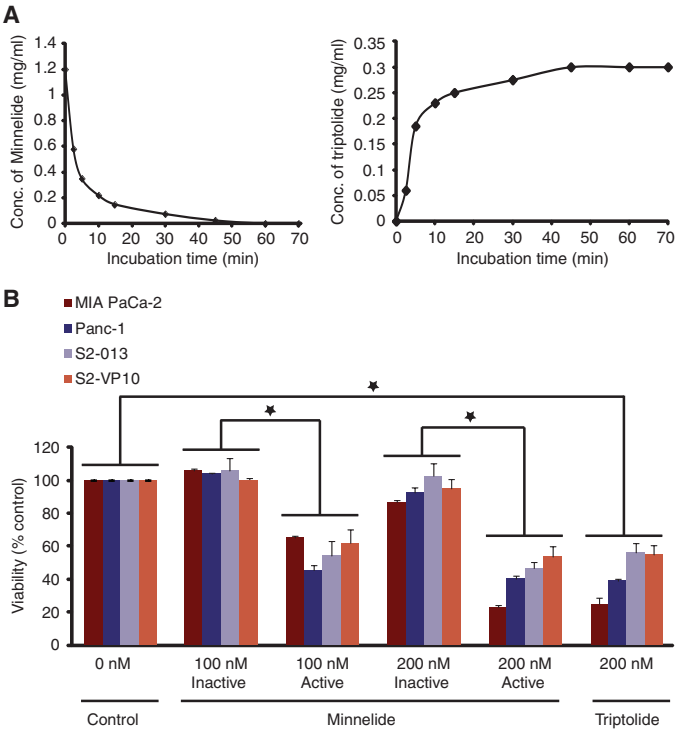
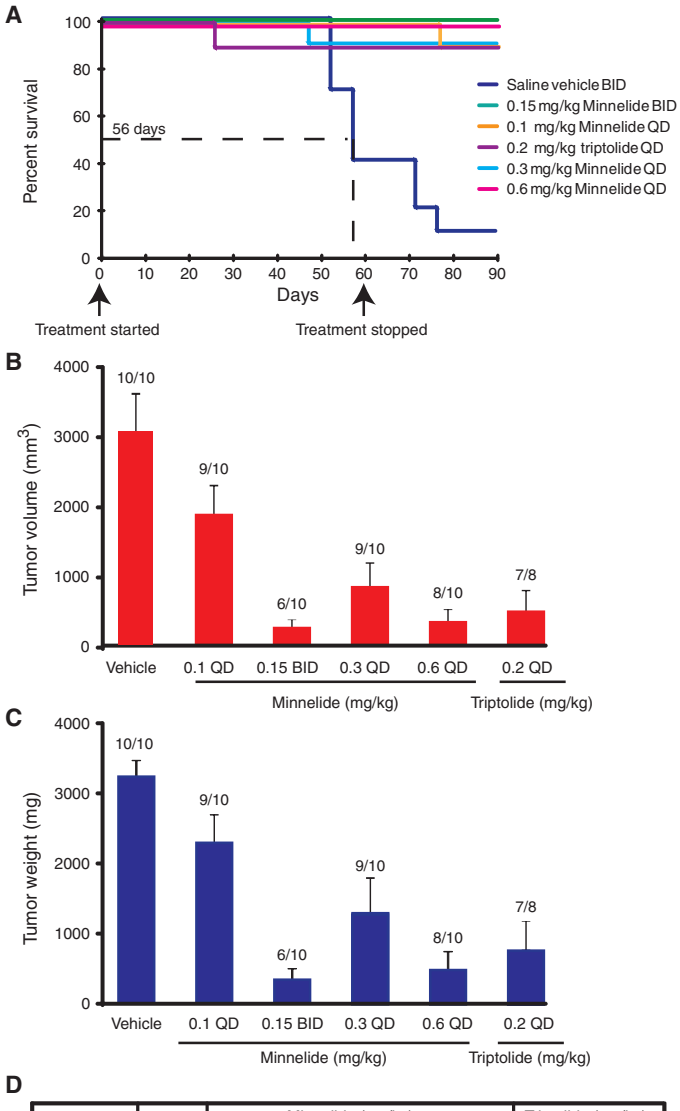


Fig. 2. Minnelide hydrolyses and decreases cell viability in pancreatic cancer cell lines. (**A**) In vitro enzymatic hydrolysis of Minnelide was performed with alkaline phosphatase in glycine buffer. The half-life of Minnelide under these conditions was 2 min. Left, degradation of Minnelide; right, generation of the parent compound, triptolide. (**B**) Pancreatic cancer cells (MIA PaCa-2, Panc-1, S2-013, and S2-VP10) were seeded in 96-well plates 24 hours before being exposed to either the inactive (without alkaline phosphatase) or the active (with alkaline phosphatase) form of Minnelide at the concentrations indicated. Cell viability was measured 48 hours after treatment and compared with untreated cells (control). Triptolide (200 nM) was used as a positive control. Statistical significance of results was calculated with the Student's *t* test. Columns, mean; bars, SE ($n = 4$; $^*P < 0.05$).



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	Saline	Minnelide (mg/kg)				Triptolide (mg/kg)
		0.1 QD	0.15 BID	0.3 QD	0.6 QD	0.2 QD
Mice alive on day 1	10	10	10	9	10	10
Mice alive on day 100	1	9	10	9	10	9

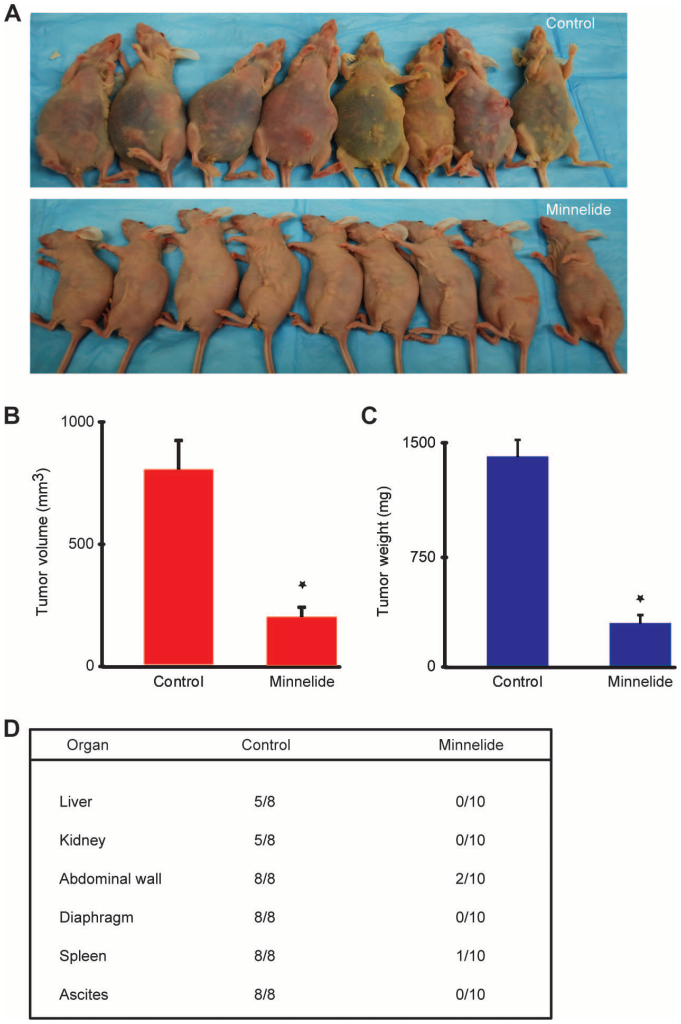
Fig. 3. Minnelide decreases pancreatic tumor growth in vivo and increases survival. MIA PaCa-2 cells (1×10^6) were injected into the tail of the pancreas of athymic Ncr nu/nu mice. (**A**) Kaplan-Meier analysis of animals injected with either triptolide (0.2 mg/kg), Minnelide (0.1 to 0.6 mg/kg QD or 0.15 mg/kg BID), or saline daily for 60 days; experiment was terminated on day 90. Treatment was started on day 12 after surgery. (**B** and **C**) Analysis of tumor volume (B) or tumor weight (C) from animals in (A). Numbers above columns represent number of animals that developed tumors over the number of animals studied. Columns, mean; bars, SE. (**D**) Table represents animals alive at start of treatment and end of experiment in each group indicated.

aminotransferase (ALT) or bilirubin levels in either males or females when compared to control animals. However, in males, the difference in ALT levels, but not in bilirubin levels, was significant at a dose of 0.6 mg/kg QD (fig. S2).

Minnelide prevents locoregional spread in a highly metastatic S2-013 cell line–derived orthotopic pancreatic cancer model

The MIA PaCa-2 cell line used in the experiment described above is derived from a human primary pancreatic tumor (*10*). However, the S2-013 cell line has been derived from a liver metastasis of pancreatic cancer (*11*, *12*). In vivo, this cell line is extremely aggressive, metastasizes rapidly, and is poorly differentiated (*12*). To evaluate the efficacy of Minnelide in a more aggressive model, we injected 1×10^6 S2-013 cells into the pancreatic tail of athymic nude mice. Because tumor induction was expected to be rapid and aggressive, treatment was started on day 7 after cell implantation at a dose of 0.42 mg/kg QD delivered intraperitoneally ($n = 8$). Tumors grew rapidly in the control saline-injected mice ($n = 8$), necessitating the termination of the experiment on day 28. Gross analysis of the control mice demonstrated the presence of ascites, jaundice, and multiple tumors, and metastasis was observed in all animals (Fig. 4). Conversely, Minnelide-injected mice did

not have ascites and did not show extensive spread from the primary site of injection (abdominal wall tumors in 2 of 10 animals and a splenic metastasis in 1 of 10 animals; Fig. 4, A and D). All control mice developed pancreatic tumors, whereas 9 of 10 Minnelide-treated animals had small tumors. Further analysis of the pancreatic tumors showed that the saline-injected mice had an average tumor volume of 799.6 ± 142.3 mm^3 compared to 199.8 ± 49.2 mm^3 in the Minnelide-treated mice (P = 0.0005). Minnelide treatment led to a decrease in average tumor weight from 1387.5 ± 109.3 mg in saline-injected mice to 290 ± 58.6 mg ($P = 6.8 \times 10^{-8}$) in Minnelide-treated mice (Fig. 4, A to C).



D

Organ	Control	Minnelide
Liver	5/8	0/10
Kidney	5/8	0/10
Abdominal wall	8/8	2/10
Diaphragm	8/8	0/10
Spleen	8/8	1/10
Ascites	8/8	0/10

Fig. 4. Minnelide decreases metastatic spread and pancreatic tumor growth. S2-013 cells (1×10^6) were injected into the tail of the pancreas of athymic Ncr nu/nu mice on day 1. On day 7 after surgery, mice were randomized, and each group of 10 mice was intraperitoneally injected with Minnelide (0.42 mg/kg) or saline daily for 28 consecutive days. (**A**) Pictorial representation of control and Minnelide-treated mice. All mice in the control group, but none in the Minnelide-treated group, displayed ascites, metastasis, and jaundice. (**B** and **C**) Graphs showing significantly reduced pancreatic tumor volume (B) and weight (C) in the Minnelide-treated group compared with the vehicle-treated group. *$P < 0.05$. (**D**) Table showing the macroscopic analysis of metastasis in the control and Minnelide-treated groups.

Minnelide prevents tumor formation and causes tumor regression in the highly metastatic AsPC-1 cell line–derived pancreatic cancer orthotopic model

To confirm our findings and to rule out potential cell line–specific effects, we established another pancreatic orthotopic mouse model using the highly metastatic ascites-derived AsPC-1 cell line (*13*). AsPC-1 cells proliferate more rapidly than S2-013 cells and are poorly differentiated (*13*). Tumor cells (2×10^5) were injected into the tail of the pancreas of nude mice (n = 20) on day 1. On day 7, treatment was initiated with either saline (n = 10) or Minnelide (0.42 mg/kg QD) (n = 10) delivered intraperitoneally. No palpable tumor was present at the start of treatment. The median survival of the control saline-treated group was 36 days, similar to that observed in the previous experiment, with an average tumor volume of 1181.0 ± 135.9 mm^3 and tumor weight of 1480 ± 154.1 mg at the time of sacrifice. Each animal in the control group had a palpable tumor. Minnelide-treated mice, on the other hand, had no palpable tumors on day 100. At this juncture, the Minnelide-treated cohort was split into two groups: Minnelide treatment at 0.42 mg/kg QD was continued in the first group (n = 5), whereas the second group (n = 5) received no further treatment [discontinued (D/C)]. No palpable tumors were observed in any mouse from either group up to the termination of the experiment on day 385 (Fig. 5A) [censored deaths at days 285 and 380 were not due to pancreatic cancer but were due to small outgrowth in intestines (one animal) and enlarged thyroid (one animal)]. Because other animals did not exhibit this phenotype, we do not attribute these deaths to Minnelide treatment. Average tumor burden at day 385, as evaluated at necropsy, was 100 ± 6.0 mg and 0 mg ($P = 2.78 \times 10^{-5}$ and 1.55×10^{-5}) or 100.0 ± 53.9 mm^3 and 0.0 mm^3 ($P = 7.79 \times 10^{-5}$ and 4.21×10^{-5}) for all Minnelide-treated animals (Fig. 5, B and C).

We further evaluated the ability of Minnelide to cause tumor regression in this model. In a design similar to that described above, we initiated treatment with Minnelide on day 28 (n = 4), after the first animal was sacrificed because of pancreatic tumor burden, and terminated the experiment at day 75. At the start of treatment, all animals had large palpable tumors (n = 9). Median survival was 36 days in the control group (n = 5), whereas all animals in the treatment group were alive at the end of the experiment (Fig. 5D). Average tumor weight in the control group was 1380.0 ± 280 mg compared to Minnelide-treated animals (50.0 ± 36.5; P = 0.01), and average tumor volume in the control group was 1301 ± 179.8 mm^3 compared to Minnelide-treated animals (18.08 ± 13.0 mm^3) (P = 0.0011).

Our data so far demonstrated that Minnelide administration could not only prevent tumor progression but also induce tumor regression, reduce metastasis, and increase survival in vivo in three pancreatic cancer cell line–based models with differing metastatic potentials.

Minnelide decreases tumor burden in a human xenograft model of pancreatic cancer

Cell line–derived tumors, although useful for prescreening of potential chemotherapeutic agents, have the disadvantage of accumulated mutations and selection pressure over time in culture, making them genetically different from the original tumors (*14*). To rigorously evaluate the ability of Minnelide to act as a chemotherapeutic agent against pancreatic cancer, we tested its effect on human pancreatic cancer xenografts. De-identified patient pancreatic adenocarcinoma tumors (n = 2) were expanded in severe combined immunodeficient (SCID) mice and, upon reaching a size of 500 mm^3, were excised from the animal, and 10-mm^3

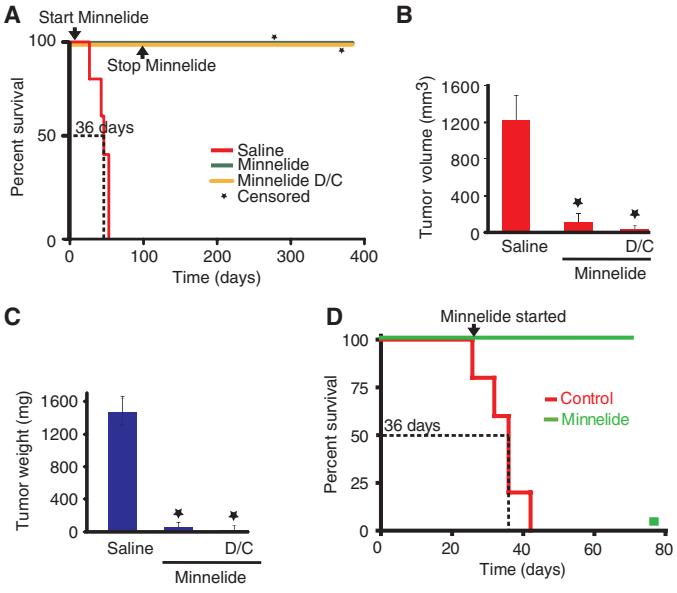


Fig. 5. Minnelide increases percent overall survival in an orthotopic xenograft mouse model. AsPC-1 cells (2×10^5) were injected into the tail of the pancreas of athymic Ncr nu/nu mice on day 1. Asterisk, censored because deaths were unrelated to pancreatic cancer. (**A**) On day 7 after surgery, mice were randomized and each group of 10 mice was intraperitoneally injected with Minnelide (0.42 mg/kg) or saline daily for 100 consecutive days. Saline-treated mice were euthanized when moribund. On day 100, the Minnelide-treated cohort (n = 10) was divided into two groups. One group (n = 5) continued to receive Minnelide (0.42 mg/kg), whereas treatment was terminated in the second group (n = 5; D/C; Stop Minnelide). Kaplan-Meier curve of the saline- or Minnelide-treated mice. Saline-treated mice had a mean survival of 36 days. All the animals in the Minnelide-treated group were alive at day 385 when the experiment was terminated. Survival lines are superimposed. (**B** and **C**) Graphs showing the average tumor volume (B) or tumor weight (C) of saline- or Minnelide-treated mice. (**D**) AsPC-1 cells (2×10^5) were injected into the tail of the pancreas of athymic Ncr nu/nu mice on day 1, and treatment with Minnelide (0.42 mg/kg) or saline was started after the death of the first animal on day 28. Animals in the saline group were sacrificed when moribund, and the experiment was terminated on day 75. Kaplan-Meier curve of the saline- or Minnelide-treated mice shows an increase in survival of Minnelide-treated animals. The median survival of saline-injected animals was 36 days.

pieces were implanted into both flanks of SCID mice (n = 20 animals). Tumors were at passage 3 when used for evaluation of Minnelide. Minnelide treatment (0.42 mg/kg; n = 10) was started when tumor size reached an average of 334.9 ± 52.8 mm^3 (day 60; Fig. 6A). The drug was delivered intraperitoneally, and as expected, saline-injected animals exhibited tumor progression. Animals exceeding allowable tumor burden were sacrificed in accordance with animal care guidelines. However, tumors regressed in Minnelide-treated animals such that, by day 40 after the start of treatment, no measurable tumors were present (Fig. 6A). Treatment was discontinued at day 50, and animals were observed for renewed tumor growth until termination of the experiment at day 120. No recurrence of tumor was observed until this point. Decrease in tumor weight corresponding to decrease in tumor volume was observed in the treatment group (90 ± 78 mg) compared with the control group (1160 ± 265 mg) (Fig. 6C). This decrease in tumor weight and volume translated into an increase in survival in the treatment group (Fig. 6B). Deaths unrelated to pancreatic cancer (four animals died of thymomas) are censored and marked by asterisks in the figure. Images of the animals at the termination of the experiment (for the treatment group) or day of sacrifice (for control animals) are shown in Fig. 6D. Histological assessment of tumors from saline-treated animals showed loss of architecture and presence of hyperchromasia and hypercellularity. However, remnants of tumors from Minnelide-treated animals showed greater fibrosis (Fig. 6E), suggesting that Minnelide causes tumor regression. The above experiment demonstrated that 0.42 mg/kg QD was an effective dose to treat xenografted human pancreatic tumors (also see fig. S1). We also tested the ability of lower doses of Minnelide to cause tumor regression in passage 4 of this xenograft model. In this experiment, treatment with Minnelide (0.21 or 0.42 mg/kg QD) was started when tumor volume reached 1000 mm^3 (1051.7 ± 141.5 mm^3). Either concentration of Minnelide was able to decrease tumor volume, although the rate at which the decrease occurred for the dose of 0.21 mg/kg was lower (days to nonmeasurable tumors, 50 versus 35 for the dose of 0.42 mg/kg; Fig. 6F). When compared with the control group, increased survival was observed in the treatment cohorts (Fig. 6G), and the experiment was terminated on day 90. Tumor weight decreased from 1737.5 ± 385.9 mg to 350 ± 25.0 mg (0.21 mg/kg) or 100 mg (0.42 mg/kg) in the treatment groups (Fig. 6H). There was no significant difference in tumor weight between the two treatment groups. Images of the animals at the termination of the experiment (for the treatment groups) or day of sacrifice (for control animals) are presented in Fig. 6I. Hematoxylin and eosin (H&E) staining of tumors from saline-treated animals showed the presence of hypercellularity and loss of architecture, whereas tumors from Minnelide-treated animals showed fibrosis and the presence of pyknotic nuclei (Fig. 6J). These data demonstrate that lower doses of Minnelide are also effective in reducing tumor burden in human xenografts from patients.

Minnelide prevents tumor formation in spontaneous pancreatic tumor–forming animals

In pancreatic cancer, the tumor microenvironment plays an important role in cancer progression; an abundant desmoplastic stroma penetrates and envelopes the tumor, comprising up to 80% of the gross tumor mass (*15*–*17*). Within the pancreatic cancer microenvironment, several cell types have been shown to affect tumor behavior (*18*, *19*), and immune response plays an important role in increasing the metastatic potential of the cancer (*20*). Because the cell line–derived models tested above lack stroma, and the human xenografts were implanted into immunodeficient animals, we tested the efficacy of Minnelide therapy in an immunocompetent animal that forms spontaneous pancreatic tumors. The $KRas^{G12D}$; $Trp53^{R172H}$; Pdx-1Cre animal model has a 100% penetrance and a median survival of 5 months (*21*). Minnelide treatment delivered intraperitoneally (0.3 mg/kg per day) was started on these animals at 4 to 6 weeks of age because median survival is 5 months in this animal model (*21*). To evaluate the decrease in tumor burden, we sacrificed at least nine animals from each group of varying age (average age: saline-treated animals, 123 days; Minnelide-treated animals, 124 days). Tumor volume and weight of saline-treated animals averaged 1380 ± 419.3 mm^3 and 1788.9 ± 561.3 mg, respectively, whereas those of Minnelide-treated animals were 251 ± 95.4 mm^3 and 333.08 ± 118.2 mg (Fig. 7). Our data suggest that Minnelide prevents tumor formation in these animals.

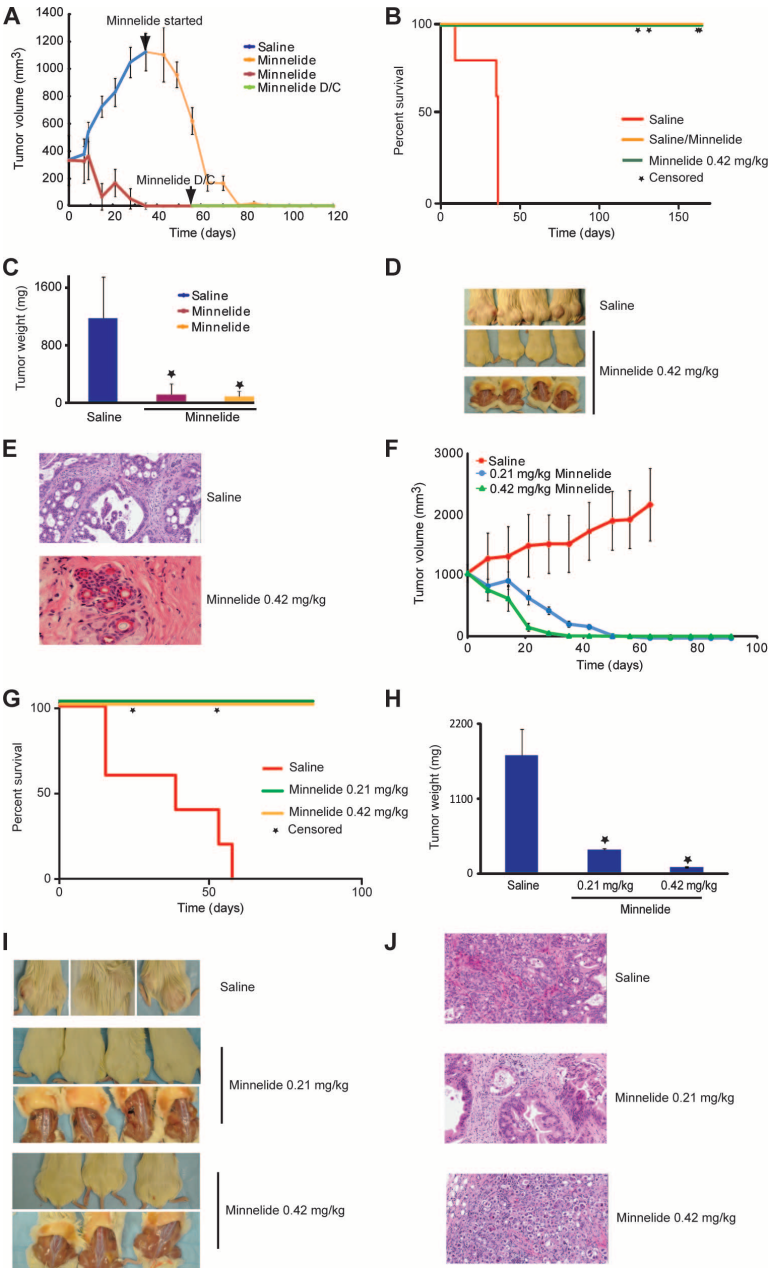


Fig. 6. Minnelide increases percent overall survival in a human xenograft model. A de-identified patient pancreatic tumor was implanted into SCID animals. (**A**) Minnelide treatment (0.42 mg/kg) was started when tumor size was either 300 mm^3 (Minnelide) or 1000 mm^3 (Minnelide). In the 300 mm^3 group, treatment was terminated on day 55 (Minnelide stop). In the 1000 mm^3 group, treatment was continued until the termination of the experiment on day 120. (**B**) Kaplan-Meier analysis of animals in (A). Asterisk, censored because deaths were unrelated to tumor burden. Saline-treated mice were euthanized when tumor size reached the limit of animal care guidelines, as described in Materials and Methods. (**C**) Tumor weight from experiment in (A). (**D**) Pictorial representation of animals in (A). (**E**) Representative H&E-stained sections from a saline-treated tumor ($n = 5$) and a Minnelide-treated tumor ($n = 11$; ×10 magnification on an Aperio ScanScope scanner). (**F**) Minnelide treatment (0.21 or 0.42 mg/kg) was started when tumor size was 1000 mm^3 (day 1). Experiment was terminated on day 90. Skin was resected to assess small subcutaneous tumors. (**G**) Kaplan-Meier survival analysis of animals in (F). Asterisk, censored because deaths were unrelated to tumor burden. Animals in the saline group were euthanized when tumor size reached the limit of animal care guidelines. (**H**) Tumor weight from experiment in (F). (**I**) Pictorial representation of animals in (F). (**J**) Representative H&E-stained sections from a saline-treated tumor ($n = 5$) and Minnelide-treated tumors [0.21 mg/kg ($n = 8$) or 0.42 mg/kg ($n = 5$)] (×10 magnification on an Aperio ScanScope scanner).

Gemcitabine is relatively ineffective in preventing pancreatic tumor formation in an orthotopic animal model of pancreatic cancer

The current first-line chemotherapy for pancreatic cancer is gemcitabine. Therefore, we compared the ability of Minnelide to prevent tumor progression to that of gemcitabine using a MIA PaCa-2 orthotopic mouse model similar to that described in Fig. 3. Tumor cells (1×10^6) were injected into the tail of the pancreas, and treatment was started 12 days after inoculation. Mice in each cohort ($n = 10$ for each) were injected intraperitoneally with either saline, Minnelide (0.28 mg/kg QD), or gemcitabine [100 mg/kg twice per week (BIW)] for 60 days. The concentration of gemcitabine used falls within the range of that evaluated previously by other groups (*22*). Although the average tumor volume was 1437.5 ± 451.2 mm^3 in control mice and 1371.4 ± 95.4 mm^3 in gemcitabine-treated mice at day 60 ($P = 0.8967$, control versus gemcitabine), it was 587.5 ± 127.4 mm^3 in Minnelide-treated mice ($P = 0.00842$, control versus Minnelide; Fig. 8A). Similarly, the average tumor weight in the control group was 2150 ± 578.17, whereas that in the gemcitabine group was 1371.4 ± 128.6 mg ($P = 0.24$, control versus gemcitabine) and that in the Minnelide group was 512.5 ± 120.2 mg ($P = 0.01495$, control versus Minnelide; Fig. 8B). Here, gemcitabine treatment did not provide a significant advantage over the control group, whereas Minnelide treatment significantly decreased tumor burden, thereby suggesting that Minnelide is more effective than gemcitabine against pancreatic cancer in this model.

DISCUSSION

Pancreatic adenocarcinoma is one of the most lethal human malignancies, with an extremely poor 5-year survival rate. Current standard of care includes treatment with gemcitabine or 5-FU, which only provides minimal survival benefit to the patient. Previous studies by our group and others have shown that triptolide, an anti-inflammatory natural compound from *T. wilfordii*, is extremely effective in inducing cell death in a number of cancer cell lines, including pancreatic cancer, in vitro. However, the use of triptolide in vivo has

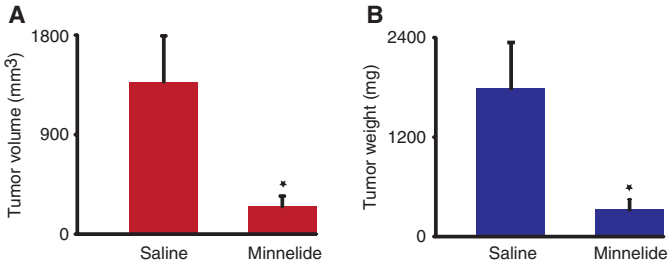


Fig. 7. Minnelide decreases tumor burden in a spontaneous pancreatic cancer mouse model. $KRas^{G12D}$; $Trp53^{R172H}$; Pdx-1Cre animals were genotyped and randomly assigned to either the saline or the Minnelide treatment group (0.3 mg/kg) at 4 to 6 weeks of age. Saline animals were sacrificed when moribund. (**A** and **B**) Tumor volume (A) and weight (**B**) of animals in either group.

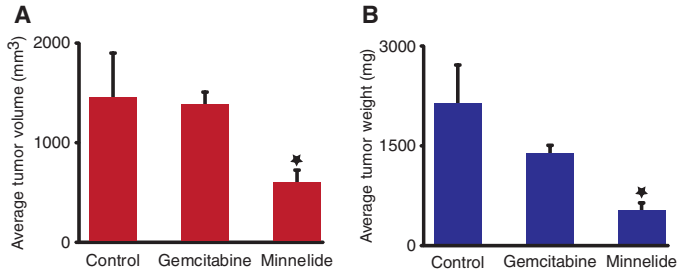


Fig. 8. Comparison of Minnelide and gemcitabine in an orthotopic pancreatic cancer mouse model. MIA PaCa-2 cells (1×10^6) were injected into the tail of the pancreas of athymic Ncr nu/nu mice. On day 12, animals were injected with Minnelide (0.28 mg/kg QD), gemcitabine (100 mg/kg BIW), or saline daily for 60 days, and experiment was terminated on day 90. (**A** and **B**) Graphs show significantly reduced (A) pancreatic tumor weight and (B) volume in Minnelide-treated mice compared with controls, whereas there is statistically no difference between gemcitabine-treated mice and controls. Columns, mean; bars, SE. *$P < 0.05$.

remained a challenge because of limited solubility in water. Here, we have shown that Minnelide, a water-soluble analog of triptolide, is very effective in decreasing tumor burden, tumor-associated morbidity, and locoregional spread in multiple animal models of pancreatic cancer. Furthermore, Minnelide is more effective than the chemotherapeutic agent gemcitabine, the current standard of care for pancreatic cancer. These results propose that Minnelide could emerge as a novel chemotherapeutic agent for the treatment of pancreatic cancer, a disease where none of the available treatments have made any significant impact.

Because pancreatic cancer is a heterogeneous disease (*23*), we have evaluated the efficacy of Minnelide in multiple complementary models, including an orthotopic model where established pancreatic cancer cell lines are injected into the tail of pancreas, a human xenograft model where human pancreatic tumors are implanted in subcutaneous tissue of mice, and state-of-the-art genetic models. In the orthotopic model, we used cell lines derived from pancreatic tumors of varying aggressiveness to simulate a range of stages of pancreatic cancer at presentation; these included cells derived from primary (MIA PaCa-2), metastatic (S2-013), and ascitic (AsPC-1) disease. To further enhance the validity of this study, we used multiple scenarios in animal models to recapitulate various patient presentations and treatment strategies, including allowing tumors to grow for variable time periods before treatment initiation. The results obtained with these complementary but independent models are in agreement, strongly suggesting that they have an increased likelihood of holding true in clinical studies.

We have conducted extensive evaluation of Minnelide in an orthotopic model of pancreatic cancer. Administration of Minnelide led to decreased tumor weight and volume in mice orthotopically injected with MIA PaCa-2 cells; tumors did not recur after termination of treatment. Similar results were observed in orthotopic mouse models generated from the metastatic pancreatic cancer cell line S2-013 and AsPC-1, where Minnelide was able to prevent or greatly reduce tumor formation and spread. To evaluate whether Minnelide suppressed tumor formation or eliminated it, we conducted survival studies using the AsPC-1 mouse model where the treatment was stopped and the animals were observed for the recurrence of tumor. We observed that Minnelide administration for 100 days resulted in a significant increase in life span [36 days in control animals versus at least 385 days (experiment termination) in treated animals], and the tumors did not return on the termination of treatment.

Our studies suggest that Minnelide is more effective when compared to other potential therapeutics against pancreatic cancer, which have been tested in mouse models. Treatments with a Smac mimetic in combination with gemcitabine led to an increase in median survival from 62 days in the control group to 122 days in the treatment group (less than twofold increase in median survival) (*24*) when treatment was initiated 28 days after tumor cell implantation. However, in this study, only 25% of the treated animals were alive on day 225. Increased median survival (105 days for treated versus 50 days for untreated MIA PaCa-2 tumor–bearing animals) was reported with a combination of gemcitabine and interleukin-13–*Pseudomonas* exotoxin (*25*). In yet another study, using the death receptor-5 agonistic antibody in combination with irinotecan, DeRosier *et al.* (*26*) show an increase in median survival of orthotopic pancreatic tumors from 76 days in the control group to 169 days in the treatment group. In contrast to the results reported in these studies, we observed 100% survival at day 385 with Minnelide monotherapy in the ascites-derived AsPC-1 animal model, compared with the median 36 days in untreated animals. The clinical reality of pancreatic cancer is that most patients are diagnosed with locally advanced or distal metastatic, surgically unresectable disease. To simulate such a clinical scenario, in one of our models, we allowed tumors derived from the aggressive AsPC-1 cell line to progress for 28 days before the initiation of treatment, at which point one animal had to be sacrificed owing to tumor burden and all animals had palpable tumors. In this context, all treated animals survived to experiment termination (day 75), whereas median survival for untreated animals was 36 days.

Although the orthotopic model using established human pancreatic cancer cell lines offers multiple advantages including the benefit of evaluating the efficacy of therapies in a natural milieu and recapitulation of locoregional spread and aggressive phenotype, there are some unique pitfalls to the use of this model. One of the criticisms leveled against the cell line–based orthotopic model has been that the cell lines may have changed over time and thus may not accurately represent human disease (*27*). To allay those concerns, we conducted a crossover study using a human xenograft model. Using primary pancreatic ductal adenocarcinoma tumor xenografts from de-identified patient samples, we demonstrated that, irrespective of initial tumor size (300 or 1000 mm^3) or Minnelide dose (0.21 or 0.42 mg/kg), tumors were

significantly ablated or abrogated in treated animals. Although tumors derived from only two different patient samples were evaluated, they both responded in a similar manner to Minnelide, suggesting that Minnelide may be effective across a range of patients. Also, this model can allay the concerns that the effect of Minnelide observed in other models is due to its localized effect when delivered intraperitoneally; in the human xenograft model, Minnelide is injected intraperitoneally and is still able to decrease the subcutaneous xenografts tumors. Again, our results in this model compare favorably with other studies evaluating novel therapeutic strategies for pancreatic cancer in human xenograft model. In a previous study with human xenografts, NanoCurc, a nanoparticle-based therapy, was able to decrease tumor volume but not completely abrogate the tumors (*28*).

On the basis of our knowledge of the heterogeneity and complexity of human tumors (*29*), the use of immunocompromised mice as preclinical models of pancreatic cancer may not accurately mimic the situation in humans. Lack of immune surveillance in these mice also prevents the recapitulation of human tumor growth. We therefore used a spontaneous cancer mouse model wherein the animal expresses $KRas^{G12D}$ and $Trp53^{R172H}$ in the pancreas [KPC animals (*21*)]. Gemcitabine has previously been shown to be ineffective in this model (*30*, *31*). However, Minnelide treatment significantly decreased tumor weight and volume in these animals (Fig. 7). Together, our data suggest that irrespective of the timing of therapy initiation or the model system used, Minnelide can inhibit the growth of pancreatic tumors and also induce regression.

We have also compared the efficacy of Minnelide with the current first-line FDA-approved chemotherapy for pancreatic cancer, gemcitabine, using the orthotopic MIA PaCa-2–derived pancreatic cancer model. Both Minnelide and its parent compound, triptolide, significantly decreased tumor weight and volume, whereas gemcitabine treatment (100 mg/kg BIW), in our hands, did not lead to a significant decrease in tumor size versus control ($P = 0.24$), underscoring the greater efficacy of Minnelide in preventing tumor growth. Our results with gemcitabine agree with previous literature showing that in MIA PaCa-2 cell–derived tumors, neither a dose of 150 or 300 mg/kg once a week for 3 weeks (*32*) nor 120 mg/kg once a week (*26*) or 80 mg/kg every third day for 45 days (*33*) revealed any significant decrease in tumor volume. In another study, MIA PaCa-2–derived tumors did not show any response to gemcitabine at a dose of 120 mg/kg BIW for the first 30 days, but there was a significant decrease in tumor volume after 35 days of treatment. Intriguingly, in other cell line–derived tumors such as SW1990 (*34*) and SUIT-2 (*35*), administration of gemcitabine at 125 mg/kg for 4 weeks (SW1990) or 40 mg/kg once a week (SUIT-2) showed a significant decrease in tumor volume. These results suggest that gemcitabine is effective in some tumors but not others. However, gemcitabine has been shown to be ineffective in the immunocompetent KPC model (*30*, *31*). This has been attributed to the short half-life of the drug and hypovascularity of pancreatic tumors, limiting the ability of gemcitabine to perfuse the tumor.

Here, we have attempted to address the limitations imposed by various preclinical models. Immortalized cell lines derived from tumors of varying metastatic potential, although representing the simplest system to assess the efficacy of a compound, may have accumulated mutations over time and lack stroma (*14*). Primary human xenografts, although less standardized, are closer to the original tumor and permit evaluation for treatment efficacy in a model that recapitulates tumor-microenvironment interactions important for progression in the clinical setting (*14*). However, the tumors are grown in immunocompromised animals lacking a systemic immune response to the tumor. The only immunocompetent model that accurately mimics human disease progression is the KPC mouse model of pancreatic cancer (*21*, *36*). The disadvantage of these animals is that they develop murine pancreatic tumors. Therefore, a preclinical study including all animal models presented here permit a more comprehensive overview of drug efficacy. However, the limitation of our study is that this compound has not been tested in humans. As several preclinical studies have demonstrated, efficacy of a compound in preclinical settings, although a required step toward human use, cannot predict the outcome of a clinical trial. In addition, no overt signs of toxicity were observed when animals received Minnelide for 385 days at a concentration of 0.42 mg/kg.

In conclusion, we have shown that Minnelide is effective in decreasing tumor burden, tumor-associated morbidity, and locoregional spread in multiple animal models of pancreatic cancer. Furthermore, we have also shown that Minnelide is more effective in preclinical studies than gemcitabine, which is the first-line chemotherapeutic agent for pancreatic cancer. Our data suggest that Minnelide has the potential to emerge as an effective therapy against not only pancreatic cancer but also many other cancers. In this regard, the phase 1 trial of Minnelide against pancreatic cancer is currently being planned and is scheduled to start by December 2012 (pending FDA approval).

MATERIALS AND METHODS

Cell lines and reagents

Pancreatic cancer cells (MIA PaCa-2, AsPC-1, and Panc-1) were obtained from the American Type Culture Collection. S2-013 cells were a gift from M. Yamamoto (University of Minnesota). Minnelide was synthesized in the Department of Medicinal Chemistry, University of Minnesota, as described in Results. Triptolide was purchased from Calbiochem, and gemcitabine was from Eli Lilly. Triptolide was resuspended in DMSO (Sigma).

Cell line maintenance

MIA PaCa-2 and Panc-1 cells derived from primary tumors of patients were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. S2-013 and S2-VP10 cells derived from patients with liver metastases were cultured in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin. AsPC-1 cells derived from patient ascites were cultured in RPMI 1640 with 20% FBS and 1% penicillin-streptomycin. All cell lines were maintained at 37°C in the presence of 5% CO_2. All cell culture reagents were obtained from Hyclone.

Cell viability

Cell viability was determined with the Dojindo Cell Counting Kit-8 (Dojindo Molecular Technologies) following the manufacturer's instructions. After treatment with triptolide or Minnelide for the times and concentrations indicated, cell viability was assessed by incubation with 10 μl of tetrazolium substrate for 1 hour at 37°C, followed by measurement of absorbance at 450 nm.

Orthotopic pancreatic cancer tumor model

Four- to 6-week-old female athymic mice (The Jackson Laboratory) were anesthetized with xylazine/ketamine and injected with 1×10^6 (MIA PaCa-2 or S2-013) or 2×10^5 (AsPC-1) cells embedded in

Matrigel (BD Biosciences) in the tail of the pancreas. Buprenorphine was used as an analgesic after surgery. Tumors were allowed to grow, and treatment of animals with either compound or control was started after animals were randomized at the times after injection stated in the figure legends. Tumor volume was calculated with the following formula: length × width × thickness/2 (*37*). Experiments were performed and animals were sacrificed in accordance to the regulations of the animal care committee of the University of Minnesota.

Human xenograft pancreatic cancer tumor model

De-identified human pancreatic tumors were implanted subcutaneously into SCID mice (The Jackson Laboratory). When tumor volumes reached 500 mm^3, tumors were dissected and cut into 10-mm^3 pieces, which were then subcutaneously implanted into both flanks of additional SCID mice (n = 20 animals). Animals were randomized and tagged before treatment, and each tumor was measured as indicated in the figures.

Transgenic mouse model of spontaneous pancreatic cancer

$KRas^{G12D}$; $Trp53^{R172H}$; Pdx-1Cre animals were generated by crossing Lox Stop Lox (LSL) $KRas^{G12D}$; LSL $Trp53^{R172H}$ animals with Pdx-1Cre animals. Minnelide treatment was started when animals were 4 to 6 weeks of age. Animals in saline and treatment groups were age-matched. Experiments were performed and animals were sacrificed in accordance with animal care committee regulations of the University of Minnesota.

Statistical analysis

Values are expressed as means ± SEM. All in vitro experiments were performed at least three times. The significance of the difference between any two samples was analyzed by independent Student's t test; P values of <0.05 were considered statistically significant.

In vitro enzymatic bioconversion of Minnelide to triptolide

All experiments involving alkaline phosphatase were performed in a pH 9.8 glycine buffer at 37°C. The buffer solution contained 1 mM $ZnCl_2$, 1 mM $MgCl_2$, and 0.1 M glycine. The final pH of the buffer was adjusted to pH 9.8 with addition of a 2 N NaOH solution. The activity of the alkaline phosphatase was measured with p-nitrophenyl phosphate as the substrate according to the procedure supplied by Sigma Chemical Co. Stock solutions of alkaline phosphatase (2000 U/liter) and Minnelide (360 μM) were prepared in glycine buffer. Alkaline phosphatase stock solution (120 μl) was added to a Minnelide stock solution (200 μl) in a shaking water bath maintained at 37°C. The final concentration of alkaline phosphatase was 750 U/liter, and the final Minnelide concentration was 255 μM. The reaction was quenched and diluted with a 0.4 N acetic acid solution (100 μl) and acetonitrile (200 μl) at predetermined time points (0, 10, 20, 30, 40, and 60 min). To demonstrate that Minnelide is chemically stable during the course of the experiment, we repeated the above-described procedure in the absence of alkaline phosphatase. After being quenched, each solution was vortexed for 30 s and injected into the HPLC and assayed to estimate the time-dependent change in the concentration of Minnelide and triptolide.

SUPPLEMENTARY MATERIALS

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Fig. S1. Human tumor xenograft from patient 2.

Fig. S2. Male and female mice were injected with Minnelide for 29 days at the doses indicated, and ALT/bilirubin levels in serum were measured on day 30.

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A Preclinical Evaluation of Minnelide as a Therapeutic Agent Against Pancreatic Cancer

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Vegetation Is Good for You

Your mom always told you to eat your vegetables, but what she probably didn't tell you is that other plants can be good for you as well. *Tripterygium wilfordii*, sometimes known as the Thunder God vine, has various uses in traditional Chinese medicine. To better understand and improve upon the healing properties of this vine, the active ingredients have been isolated and characterized. One component of *T. wilfordii*, triptolide, has shown promising effects against pancreatic cancer cells. New therapies for pancreatic cancer—which is one of the most lethal human malignancies—are desperately needed, but triptolide is poorly soluble in water and thus has limited clinical use. Now, Chugh *et al.* synthesize a water-soluble form of triptolide, Minnelide, and demonstrate efficacy against pancreatic cancer in multiple animal models.

The authors tested Minnelide both in vitro and in multiple preclinical models of pancreatic cancer. Each model has distinct advantages and limitations: Well-studied cancer cell lines and translationally relevant patient tumors were transplanted into mice that lack immune systems, whereas a spontaneous model in immunosufficient mice was, by necessity, a mouse tumor. By combining these approaches, the authors addressed many caveats that frequently plague preclinical studies. Indeed, Minnelide was highly effective in treating pancreatic cancer in all of these complementary models. The next step is to take Minnelide into early clinical trials to see if these results can be reproduced in human patients with pancreatic cancer.