Selective Antimetastatic Activity of Cytosine Analog CS-682 in a Red Fluorescent Protein Orthotopic Model of Pancreatic Cancer

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ABSTRACT

In this study we demonstrate the ability of a novel, p.o.-administered cytosine analogue, CS-682, to effectively prolong survival and inhibit metastatic growth in an imageable orthotopic mouse model of pancreatic cancer. MIA-PaCa-2-RFP pancreatic cancer cells were transduced with the Discosoma red fluorescent protein (RFP) and orthotopically implanted onto the pancreas of nude mice. Tumor RFP fluorescence facilitated real-time, sequential imaging, and quantification of primary and metastatic growth and dissemination in vivo. Mice were treated with various p.o. doses of CS-682 on a five times per week schedule until death. At a dose of 40 mg/kg, CS-682 prolonged survival compared with untreated animals (median survival 35 days versus 17 days; P = 0.0008). At nontoxic doses, CS-682 effectively suppressed the rate of primary tumor growth. CS-682 also decreased the development of malignant ascites and the formation of metastases, which were reduced significantly in number in the diaphragm, lymph nodes, liver, and kidney. Selective RFP tumor fluorescence enabled noninvasive real-time comparison between groups during treatment and facilitated identification of micrometastases in solid organs at autopsy. Thus, we have demonstrated that CS-682 is an efficacious antimetastatic agent that significantly prolongs survival in an orthotopic model of pancreatic cancer. The antimetastatic efficacy of CS-682 and its p.o. availability confer significant advantages and clinical potential to this agent for pancreatic cancer.

INTRODUCTION

Pancreatic ductal adenocarcinoma is one of the most lethal of human malignancies, accounting for >30,000 deaths yearly in the United States alone (1). On diagnosis, only 10–15% of these cancers are typically found to be resectable (2) due to the presence of locally advanced disease or distant metastases. Currently, the most common strategy in the treatment of advanced pancreatic cancer is treatment with gemcitabine (3), an i.v.-administered 2′-deoxycytidine nucleoside analogue. Gemcitabine induces apoptosis of human pancreatic cancer cells and can inhibit tumor growth and progression (3). However, despite maximal medical or surgical management, results of the treatment of patients with pancreatic ductal adenocarcinoma are dismal. Patients with this disease have a median survival <21 months (4). Clearly, new, effective treatment strategies are required to combat this deadly disease.

A novel nucleoside analogue, CS-682, has been described recently and has been shown to have potent antitumor activity in several human xenograft models (5–7). CS-682 is a p.o. administered N4-palmitoyl derivative of 1-(2-C-cyano-2-deoxy-β-D-arabinopyranosyl)cytosine (CNDAC). The antitumor effect of this 2′-deoxycytidine analogue is thought to be due to both its ability to inhibit DNA polymerase and its ability to induce DNA strand breakage through incorporation of an active metabolite into the strands (6). Notably, p.o. CS-682 has been shown to exhibit more potent cytotoxic activity than its parent compound against several tumor cell lines, including those of the stomach, lung, colon, and breast (6).

In this study, we demonstrate the ability of a novel, p.o.-administered cytosine analogue, CS-682, to effectively prolong survival and inhibit metastatic growth in an imageable orthotopic mouse model of pancreatic cancer. MIA-PaCa-2-RFP pancreatic cancer cells were transduced with the Discosoma red fluorescent protein (RFP) and orthotopically implanted onto the pancreas of nude mice. Tumor RFP fluorescence facilitated real-time, sequential imaging, and quantification of primary and metastatic growth and dissemination in vivo. Mice were treated with various p.o. doses of CS-682 on a five times per week schedule until death. At a dose of 40 mg/kg, CS-682 prolonged survival compared with untreated animals (median survival 35 days versus 17 days; P = 0.0008). At nontoxic doses, CS-682 effectively suppressed the rate of primary tumor growth. CS-682 also decreased the development of malignant ascites and the formation of metastases, which were reduced significantly in number in the diaphragm, lymph nodes, liver, and kidney. Selective RFP tumor fluorescence enabled noninvasive real-time comparison between groups during treatment and facilitated identification of micrometastases in solid organs at autopsy. Thus, we have demonstrated that CS-682 is an efficacious antimetastatic agent that significantly prolongs survival in an orthotopic model of pancreatic cancer. The antimetastatic efficacy of CS-682 and its p.o. availability confer significant advantages and clinical potential to this agent for pancreatic cancer.

MATERIALS AND METHODS

Cell Line. The MIA-PaCa-2 human pancreatic cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin and streptomycin (Life Technologies, Inc., Grand Island, NY). Cells were cultured at 37°C in a 5% CO₂ incubator.

RFP Retroviral Transduction and Selection of MIA-PaCa-2-RFP Pancreatic Cancer Cells. The pDsRed-2 vector (Clontech Laboratories Inc., Palo Alto, CA) was used to engineer MIA-PaCa-2 clones stably expressing RFP. This vector expresses RFP and the neomycin resistance gene on the same bicistronic message, and has been demonstrated to exhibit low toxicity in mammalian cell lines. pDsRed-2 was produced in PT67 packaging cells. RFP transduction was initiated by incubating 20% confluent MIA-PaCa-2 cells with retroviral supernatants of the packaging cells and DMEM for 24 h. Fresh medium was replenished at this time, and cells were allowed to grow in the absence of retrovirus for 12 h. This procedure was repeated until high levels of RFP expression, as determined using fluorescence microscopy, were achieved. Cells were then harvested by trypsin/EDTA and cultured into selective medium that contained 200 μg/ml G418. The level of G418 was increased to 2000 μg/ml stepwise. Clones expressing high levels of RFP were isolated with cloning cylinders and were amplified and transferred using conventional culture methods. High RFP-expressing clones were isolated in the absence of G418 for 10 passages to select for stable expression of RFP in vivo.

Animals. Male nude mice (NCI-nu/nu) between 4 and 6 weeks of age were maintained in a barrier facility on HEPA-filtered racks. The animals were fed with autoclaved laboratory rodent diet (Tekland LM-485; Western Research Products, Orange, CA). Animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication Number 85–23) under assurance number A3873–01.

SOI of MIA-PaCa-2-RFP Tumors. Red-fluorescent human pancreatic cancer xenografts were established in nude mice by SOI (9). Briefly, MIA-
G418 up to 2000
/H9262
pDsRed-2 retroviral vector-
MIA-PaCa-2 and MIA-PaCa-2-RFP.

Doses, body weight declined to
once daily, no decrease in body weight was noted (Fig. 1). At higher
doses were resected asexually. Necrotic tissues were cut away, and the remaining
healthy tissues were cut with scissors and minced into 1-mm³ pieces in
RPMI 1640. Mice were then anesthetized, and their abdomens were sterilized
with alcohol. An incision was then created through the left upper abdominal
pararectal line and peritoneum. The pancreas was carefully exposed, and two
tumor pieces were transplanted onto the middle of the gland using a single 8–0
surgical suture (Davis-Geck, Inc., Manati, Puerto Rico). The pancreas was then
returned into the peritoneal cavity, and the abdominal wall and the skin were
closed in two layers using 6–0 surgical sutures. All of the procedures were
performed with a ×1 microscope (Olympus) or standard surgical loupes.

**Drug Dose, Route, and Schedule.** CS-682 [1-(2-C-cyano-2-deoxy-β-D-
araibino-pentofuranosyl)-N⁴-palmitoylcysteine; Sankyo Pharmaceuticals, Tokyo] was administered by oral gavage. Before the first treatment, mice were
randomized into eight groups of 10 mice each for treatment purposes. Group
1 served as the negative control and did not receive treatment. Each scheduled
treatment day, groups 2, 3, and 4 received 40, 60, and 80 mg/kg/dose CS-682,
respectively. Groups 5, 6, 7, and 8 received 20, 30, 40, and 50 mg/kg/dose
CS-682 twice each treatment day, respectively. Dosing was initiated 5 days
after SOI and was performed 5 days each week until death.

**External in Vivo Whole Body Imaging.** At least once a week, mice were
weighed and underwent external in vivo fluorescence imaging (10). This was performed in a fluorescent light box illuminated by fiberoptic lighting at 470
nm (Lightools Research, Encinitas, CA). Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology, Battleboro, VT) on a
Hamamatsu CS810 3-chip cooled color CCD camera (Hamamatsu Photonics
Systems, Bridgewater, NJ). High resolution images of 1024 × 724 pixels were
captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR model SLV-R1000 (Sony Corp., Tokyo, Japan).
Images were processed for contrast and brightness and analyzed with the use
of Image Pro Plus 3.1 software (Media Cybernetics, Silver Spring, MD). Real-time determination of tumor burden was performed by quantifying fluo-
rescent surface area as described previously (10, 11).

**Direct Imaging and RFP Fluorescence Microscopy.** Mice were sacri-
ficed and explored when they appeared premorbid. After euthanasia, each
mice underwent laparotomy and sternotomy. Excitation of RFP in the light
box, described above, facilitated identification of primary and metastatic
disease by fluorescence visualization (12). After performing full-body, open
images, the solid organs were removed, and their surfaces were thoroughly
examined for any evidence of metastases. Organs were then frozen and sliced
into cross-sectional samples ~2 mm in width with a razor blade and visualized
through a Leica fluorescence stereo microscope model L2Z (Leica Micro-
systems, Inc., Bannockburn, IL) equipped with a mercury 50-W lamp power
supply. Selective excitation of RFP was produced through a D425/60 band-
pass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected
by the Hamamatsu camera system described above.

**Statistical Analysis.** Differences among treatment groups were assessed
using ANOVA and Student’s t test using Statistica (Statsoft, Inc., Tulsa, OK).
Kaplan-Meier analysis with a log-rank test was used to determine survival and
differences between treatment groups. A P ≤ 0.05 was considered to be
statistically significant.

**RESULTS**

**Comparison of Cell Morphology and in Vitro Growth Rates of**
MIA-PaCa-2 and MIA-PaCa-2-RFP, pDsRed-2 retroviral vector-
transduced cells were able to grow in vitro in medium containing
G418 up to 2000 µg/ml. The MIA-PaCa-2 cells selected for G418
resistance had bright RFP fluorescence that remained stable in the
absence of selective medium after numerous passages. The RFP
transductants are morphologically identical to their parental cell line
(data not shown). The growth rates of the parental cells and the RFP
transductants were found to be statistically equivalent (data not shown).

**Body Weight Loss and Toxicity.** At a CS-682 dose of 40 mg/kg
once daily, no decrease in body weight was noted (Fig. 1). At higher
doses, body weight declined to <80% of baseline. At these doses,

**Survival.** Median survival of untreated mice with MIA-PaCa-2-
RFP pancreatic cancer was 17 days. p.o. administration of CS-682
significantly prolonged survival at several doses (Fig. 2). This effect
was most significant at 40 mg/kg (median survival 35 days; P = 0.0008).
Significance was also seen at doses of 60 mg/kg (median survival 36 days; P = 0.002) and 20 mg/kg twice daily (median survival 27 days; P = 0.003). In the latter two groups, a significant
increase in survival was achieved despite a loss of several mice in
each group to drug-associated toxicity. Prolongation of survival was
not significantly different between these three treatment groups
(P = 0.19). At twice-daily doses, survival was not enhanced by
CS-682 administration, with a median survival of 18 days at a dose of
50 mg/kg twice daily, and 19 days at doses of 30 mg/kg and 40 mg/kg
twice daily, as well as 80 mg/kg daily.

**Real-Time Imaging of Tumor Growth and Metastasis, and
Quantification of CS-682 Efficacy.** Selective tumor RFP fluores-
cence enabled real-time, sequential whole-body imaging and quanti-
fication of tumor burden. In control mice, significant primary tumor
growth and metastatic spread was visible within the first 2 weeks after
SOI of tumor (Fig. 3A). On day 16 after SOI, each of these mice was
visualized by whole-body imaging of RFP fluorescence to have dis-
seminated metastatic disease in all four quadrants of the abdominal
cavity. Additionally, the development of malignant ascites was found
in 100% of control animals within the first 16 days after implantation.
Aliquots of this ascites fluid drawn and cultured at the time of autopsy

![Graph](image-url)
formed colonies of high-expressing RFP clones of MIA-PaCa-2-RFP cells (Fig. 4).

In contrast, mice treated with CS-682 did not demonstrate significant tumor dissemination until week 3 or 4 after implantation (Fig. 3A). By day 16, when control animals were found to have massive intra-abdominal dissemination of tumor, 90% of mice treated with 40 mg/kg daily CS-682 were found to have locally confined disease. Accumulation of ascites was also less frequent in treated animals, with 50% and 10% of mice at treatment doses of 40 mg/kg and 60 mg/kg daily, respectively, having evident intra-abdominal fluid on examination.

Quantification of RFP fluorescent area facilitated real-time comparison of each treatment dose (Fig. 3B), demonstrating the ability of CS-682 to inhibit pancreatic cancer growth at dosages of 20 mg/kg twice daily, 40 mg/kg daily, and 60 mg/kg daily (P < 0.05 at each time point).

Effect of CS-682 on Primary Tumor Growth and Metastasis. p.o. administration of CS-682 had a significant effect on the development of lymphatic, peritoneal, and solid organ metastases in this pancreatic cancer model (Fig. 5). In untreated animals at the time of autopsy, metastases were found in the spleen (100%), intestinal nodes (100%), portal nodes (90%), liver (80%), retroperitoneum (60%), diaphragm (50%), kidney (30%), and lung (10%; Fig. 6). In contrast, treatment with CS-682 at 40 mg/kg daily significantly inhibited the development of metastases in the diaphragm, portal nodes, liver, intestinal nodes, and kidney. Dosages >40 mg/kg daily additionally decreased the number of metastases found at autopsy but with increased toxicity.

Real-time imaging revealed that CS-682 inhibited the growth rate of primary pancreatic tumors at all of the doses tested. However, the increase in life span associated with a CS-682 dose of 40 mg/kg daily permitted the primary tumors in this group to ultimately grow to a size statistically similar to those in controls (Fig. 7). At the time of autopsy, primary tumors in control mice had an average weight of 5.163 g, which was statistically similar to the primary weight of those mice treated with CS-682 at a dose of 40 mg/kg (3.935 g; P = 0.16).

Treatment with higher, toxic doses of CS-682 conferred a more significant effect on primary tumor growth (60 mg/kg daily, P = 0.0004 and 20 mg/kg twice daily, P = 0.003), but these doses appeared to lose tumor selectivity and were associated with significant toxicity.

DISCUSSION

In this study, we clearly demonstrate the potent efficacy of p.o. CS-682 in the treatment of a highly malignant pancreatic cancer model. At a dose of 40 mg/kg daily, CS-682 had a significant (P = 0.0008) effect on survival and was associated with little evi-
Importantly, we have shown that the increase in survival attained with this dose appears to be attributable in large part to the ability of CS-682 to inhibit distant metastasis, which were dramatically reduced in several sites including the diaphragm, lymph nodes, liver, and kidney after treatment with this agent. The primary tumors in the CS-682 40 mg/kg-treated group ultimately grew to a size similar to controls because of the increase in life span in these animals.

The antimetastatic properties of CS-682 make it an attractive candidate for pancreatic cancer chemotherapy. Furthermore, the ability to dose the drug p.o., in contrast with the i.v. delivery of drugs used currently for the disease, implies that the drug may be better tolerated and more acceptable to patients. We have shown previously that gemcitabine, used in an orthotopic mouse model of pancreatic cancer with adjuvant treatment, has antimetastatic efficacy (13). The equivalent adjuvant use of CS-682 after surgical resection would appear to be a potential indication for this oral agent.

It should be noted that we have used a novel, RFP-expressive orthotopic model of pancreatic cancer for this study. The model uses the highly metastatic cell line MIA-PaCa-2, which has been engineered to selectively express RFP but is otherwise similar to its parental line in terms of morphology and growth characteristics. Our model is ideal for such therapeutic trials. First, the MIA-PaCa-2-RFP orthotopic model has a high degree of clinical relevance because it gives rise to a profile of metastasis consistent with that of clinical human pancreatic cancer with diffuse metastases to various intraperitoneal sites appearing early in its natural history. Second, the high level of selective RFP tumor fluorescence enables sequential whole-body imaging and quantification of tumor growth and dissemination under different therapeutic conditions. Whole-body imaging enables real-time comparison of the antitumor effects of different agents without the need for invasive procedures. Furthermore, micrometastases that might otherwise be overlooked in nonfluorescent models can be easily visualized using fluorescence microscopy instead of using cumbersome histological techniques.

A previous report on the use of CS-682 in a liver metastatic mouse model (3) showed that CS-682 administered at 40 mg/kg daily inhibited the development of ascites and metastases to the diaphragm, portal lymph nodes, liver, intestinal lymph nodes, and kidney. Increasing the CS-682 dose additionally decreased metastasis formation but increased toxicity.

**Fig. 4.** Culture of the ascites fluid from the MIA-PaCa-2-RFP model. Colonies of high-RFP expressing cells were readily recovered from the animals and cultured (magnification, ×100). All control mice developed malignant ascites within 2 weeks of SOI.

**Fig. 5.** Direct in vivo images of mice upon autopsy demonstrated diffuse lymphatic, peritoneal, and solid organ metastases in untreated animals. Panels display a representative mouse from each of three treatment groups at the time of autopsy. A, control. B, CS-682 40 mg/kg daily. C, CS-682 60 mg/kg daily. Extensive primary tumor growth, as well as metastases to the diaphragm, peritoneum, liver, and mesenteric and portal lymph nodes were evident in almost all mice in the control group. Distant metastases were less frequent in mice treated with CS-682.

**Fig. 6.** CS-682 administered at 40 mg/kg daily inhibited the development of ascites and metastases to the diaphragm, portal lymph nodes, liver, intestinal lymph nodes, and kidney. Increasing the CS-682 dose additionally decreased metastasis formation but increased toxicity.
model (7) used serial magnetic resonance imaging of tumor and metastatic spread to determine drug efficacy. Localization of intra-abdominal tumor was facilitated using this imaging modality. However, magnetic resonance imaging requires the use of i.v. anesthesia, contrast agents, and expensive technical equipment. Moreover, precise determination of solid tumor metastases in such nonfluorescent models requires the use of histological staining. Our model enables real-time imaging of internal disease without the need for expensive imaging systems, contrast agents, substrates, or anesthesia.

In summary, we have demonstrated that the p.o. administration of the nucleoside analogue CS-682 prolongs survival by inhibiting the development of metastases in an orthotopic pancreatic cancer mouse model. Additional studies are necessary to elucidate the basis of the selective antimetastatic properties of CS-682 and to demonstrate the use of this novel and promising agent in adjuvant and combination treatment preclinically and clinically.

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