[CANCER RESEARCH 63, 5521–5525, September 1, 2003]

Selective Antimetastatic Activity of Cytosine Analog CS-682 in a Red Fluorescent Protein Orthotopic Model of Pancreatic Cancer[1]

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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 tumor xenograft models (5–7). CS-682 is a p.o. administered *N*4-palmitoyl derivative of 1-(2-C-cyano-2-deoxy-β-D-arabino-pentofuranosyl) 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).

Previous studies of the effects of CS-682, although enticing, have primarily demonstrated efficacy of the drug in *in vitro* cell cultures and nonmetastasizing s.c. xenograft models. Only one prior study has described the effects of CS-682 on liver metastasis (7). Moreover, the effect of CS-682 on pancreatic cancer has not yet been demonstrated.

In this report, we examine the ability of p.o.-administered CS-682 to both inhibit metastasis and prolong survival in a novel, orthotopic model of pancreatic cancer that selectively expresses high level of *Driscosoma sp*. RFP[3] (8). This model has two important features that make it an ideal system with which to study the effects of CS-682. First, it is a highly malignant orthotopic model that, left untreated, spontaneously gives rise to extensive dissemination to the lymphatics, peritoneum, and solid organs, as well as ascites. Second, its high level of selective tumor RFP fluorescence facilitates *in vivo* visualization and quantification of tumor growth and metastasis development, and, therefore, allows real-time evaluation of the drug on pancreatic cancer without the need for laparotomy, substrates, contrast agents, or other invasive procedures.

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% CO_2 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 subcultured 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-expression clones were isolated in the absence of G418 for 10 passages to select for stable expression of RFP *in vivo*.

Animals. Male nude mice (NCr-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 (Teckland 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-

Received 4/21/03; revised 6/19/03; accepted 6/25/03.

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[1] Supported in part by the Department of Health Services, California Cancer Research Program (97-120B), and National Cancer Institute Grants P30 CA23100-1851 and R43-89779.

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[3] The abbreviations used are: RFP, red fluorescent protein; SOI, surgical orthotopic implantation.

PaCa-2-RFP tumors in the exponential growth phase, grown s.c. in nude mice, were resected aseptically. Necrotic tissues were cut away, and the remaining healthy tumor tissues were cut with scissors and minced into 1-mm^3 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 7× microscope (Olympus) or standard surgical loupes.

Drug Dose, Route, and Schedule. CS-682 [1-(2-*C*-cyano-2-deoxy-β-D-*arabino*-pentofuranosyl)-N^4-palmitoylcytosine; 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 C5810 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 fluorescent surface area as described previously (10, 11).

Direct Imaging and RFP Fluorescence Microscopy. Mice were sacrificed and explored when they appeared premorbid. After euthanasia, each mouse 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 LZ12 (Leica Microsystems, 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 $P \leq 0.05$ 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,

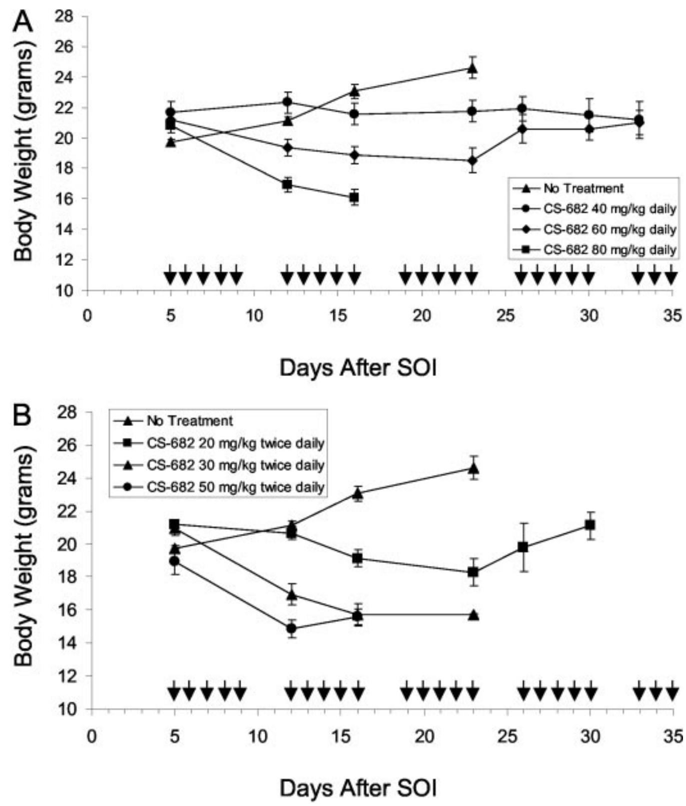

Fig. 1. p.o. CS-682 administration had little toxicity or effect on mouse body weight when administered at a dose of 40 mg/kg daily. At higher doses, body weight declined >20%. *A*, body weights with once-a-day dosing of CS-682 in mice with surgically implanted MIA-PaCa-2-RFP pancreatic tumors. *B*, body weights with twice-daily dosing. Values represent average weight of live animals in each treatment group; *bars*, ±SE.

toxicity-related death was observed in a significant number of mice. Notably, once-a-day dosing at 40 mg/kg and 60 mg/kg was associated with less weight loss and toxicity than was delivering the same dose divided twice daily.

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 fluorescence enabled real-time, sequential whole-body imaging and quantification 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. 3*A*). On day 16 after SOI, each of these mice was visualized by whole-body imaging of RFP fluorescence to have disseminated 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

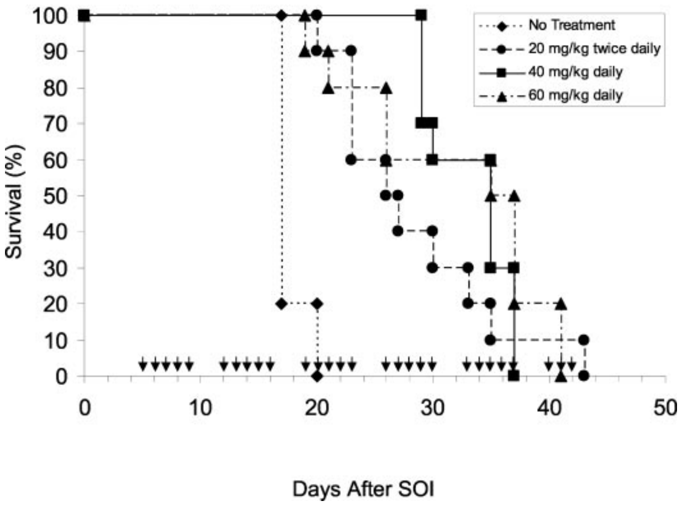

Fig. 2. p.o. administration of CS-682 significantly prolonged survival in mice with MIA-PaCa-2-RFP pancreatic cancer. Median survival was increased from 17 days in the control group to 27 days ($P = 0.003$), 35 days ($P = 0.0008$), and 36 days ($P = 0.002$) at CS-682 dosages of 20 mg/kg twice daily, 40 mg/kg daily, and 60 mg/kg daily, respectively.

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. 3*A*). 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. 3*B*), 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-

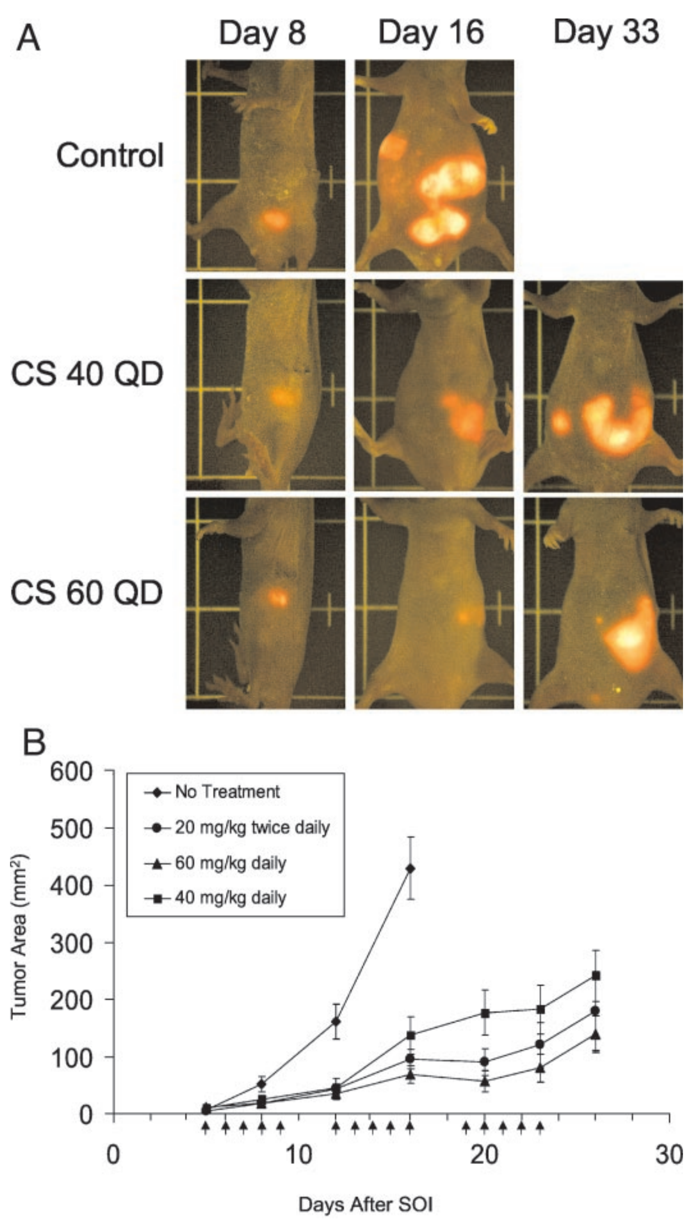

Fig. 3. *A*, sequential *in vivo* imaging of tumor progression over time in mice with MIA-PaCa-2-RFP orthotopic xenografts. Selective tumor RFP fluorescence facilitated real-time visualization of tumor burden in the live animal. *Panels* depict a representative mouse from each of three treatment groups on days 8, 16, and 33 after tumor implantation. Tumor dissemination in all four abdominal quadrants was visible within the first 2 weeks after implantation in the control group. In mice treated with CS-682 at 40 mg/kg and 60 mg/kg daily, widespread tumor metastasis was not visible until the weeks 3 and 4 postimplantation. *B*, quantification of primary and metastatic tumor RFP fluorescence enabled real-time determination and comparison of tumor load during the course of each treatment and, therefore, permitted real-time comparison of treatment efficacy between groups. In each group, progression of disease as evaluated using this method had a strong correlation to survival. Values represent the mean area of external RFP fluorescence for live intact animals in each treatment group; *bars*, ±SE ($P < 0.05$ for each time point) after day 8 compared to control.

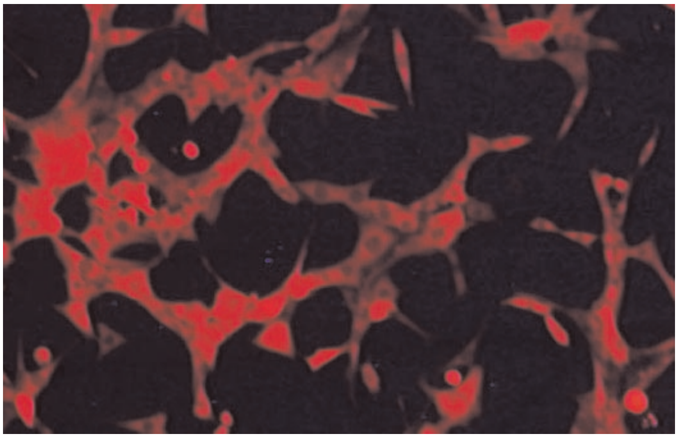

Fig. 4. Culture of the ascites fluid from the MIA-PaCa-2-RFP model. Colonies of high-RFP expressing cells were readily recovered from the animals and cultured (magnification, ×100). All control mice developed malignant ascites within 2 weeks of SOI.

dence of systemic toxicity. 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 intra-abdominal 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

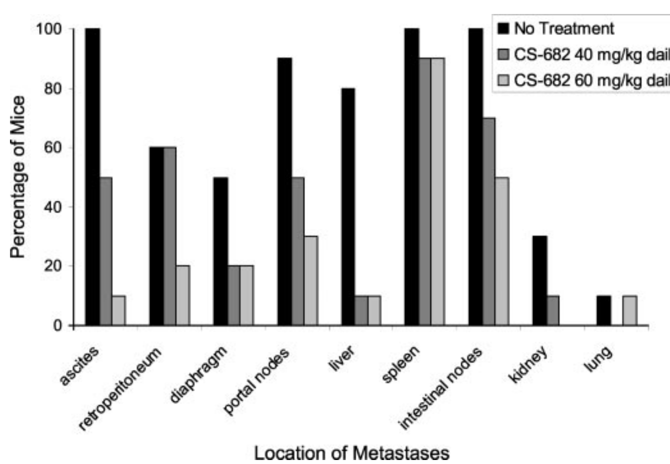

Fig. 6. CS-682 administered at 40 mg/kg daily inhibited the development of ascites and metastases to the diaphragm, portal lymph nodes, liver, intestinal lymph nodes, and kidney. Increasing the CS-682 dose additionally decreased metastasis formation but increased toxicity.

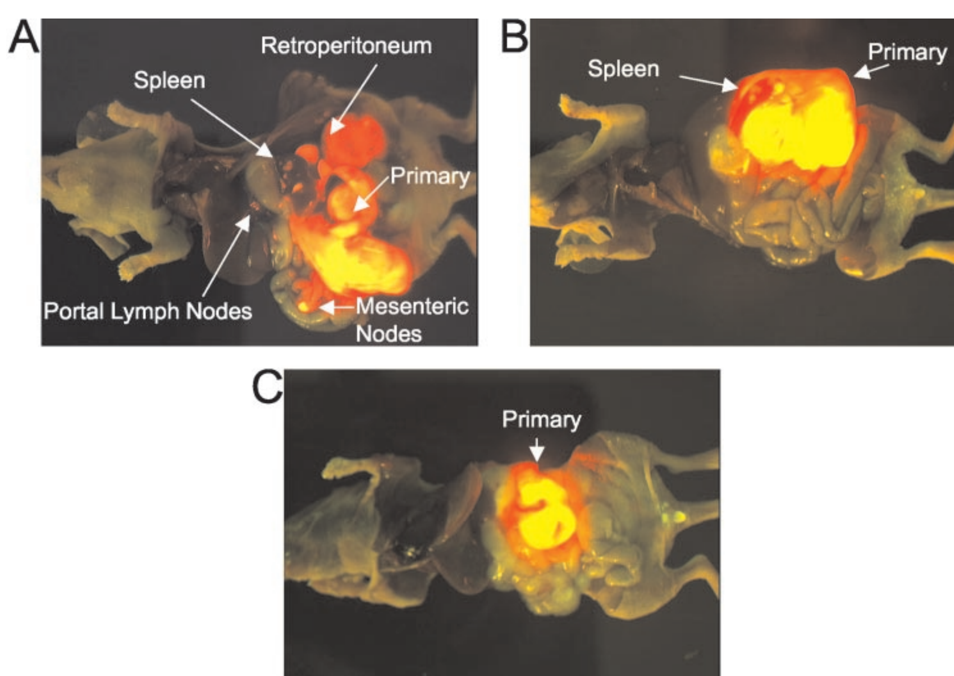

Fig. 5. Direct *in vivo* images of mice upon autopsy demonstrated diffuse lymphatic, peritoneal, and solid organ metastases in untreated animals. *Panels* display a representative mouse from each of three treatment groups at the time of autopsy. *A*, control. *B*, CS-682 40 mg/kg daily. *C*, CS-682 60 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.

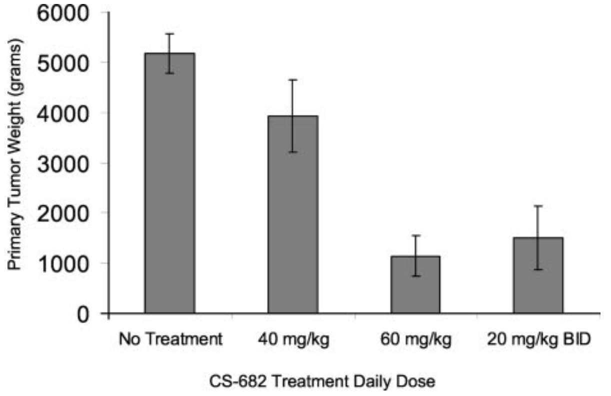

Fig. 7. At 40 mg/kg daily of CS-682, primary tumor weight at autopsy was statistically similar to that in control mice ($P = 0.16$). *Bars* represent the average weight of the primary tumors of the mice in each treatment group as determined at autopsy. Error bars, ±SE.

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.

ACKNOWLEDGMENTS

We thank the Sankyo Co., Ltd., for providing CS-682.

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