High-Resolution Magnetic Resonance Imaging of the Efficacy of the Cytosine Analogue 1-[2-Cyano-2-deoxy-β-D-arabinofuranosyl]-N4-palmitoyl Cytosine (CS-682) in a Liver-Metastasis Athymic Nude Mouse Model

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ABSTRACT

High-resolution magnetic resonance (MR) imaging techniques in a liver metastatic nude mouse model were used to assess CS-682, a novel 2′-deoxy-cytidine analogue of 1-[2-Cyano-2-deoxy-β-D-arabinofuranosyl]-N4-palmitoyl cytosine. The efficacy of CS-682 was visualized in real time by MR imaging of intact seeding and subsequent growth of liver metastases. The relative therapeutic efficacies of CS-682 and two agents used clinically, gemcitabine (2′-deoxy-2′-2′-difluorocytidine monophosphoryl chloride (DFDC)) and 5-fluorouracil (5-FU), were compared in this model. CS-682 was found to exhibit superior efficacy by delaying the onset and inhibiting the growth of liver metastases compared with gemcitabine, 5-FU, and control. The overall occurrence of metastases was decreased 62% by CS-682, 18% by DFDC, and 35% by 5-FU. CS-682 increased the life span of the treated animals significantly, by 28 days above the 29-day median survival without treatment, compared with 11 days by DFDC and 14 days by 5-FU. The increased survival in CS-682-treated animals correlated with the antitumor activity of this compound. These preclinical findings support the potential clinical utility of CS-682 in the treatment of liver metastases.

INTRODUCTION

As the main cause of treatment failure in humans, metastasis is an important and clinically relevant target for therapeutic intervention. In this report, we used high-resolution MRI techniques in a nude mouse model of preferential metastasis to the liver (1–3) to evaluate the antitumor efficacy of the pyrimidine analogue CS-682.

Many analogues of pyrimidine nucleotides have been synthesized as antitumor agents capable of inhibiting DNA and RNA synthesis, including the clinical therapeutic agents DFDC (4) and 5-FU (5), commonly used in the treatment of many types of cancers. Similar to the mechanisms of antitumor activity of other 2′-deoxy-cytidine analogues, CS-682 inhibits DNA synthesis via the inhibition of DNA polymerase (6). Additionally, CS-682 has been shown to exhibit a novel DNA self-strand-breaking activity in cell-free and intact tumor cell systems, suggesting an additional mechanism contributing to the potent antitumor activity of CS-682 (6). The N4-palmitoyl group of CS-682 is not a substrate for cytidine deaminase, which is found in abundance in tumor tissues, thus conferring protection against degra-

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3 The abbreviations used are: MR, magnetic resonance; MRI, MR imaging; CS-682, 1-[2-Cyano-2-deoxy-β-D-arabinofuranosyl]-N4-palmitoyl cytosine; DFDC, 2′-deoxy-2′-2′-difluorocytidine monophosphoryl chloride (gemcitabine); T1, longitudinal relaxation time; T2, transverse relaxation time; RARE, rapid acquisition with relaxation enhancement; SE, spin echo; MTD, maximally tolerated dose; ILS, increased life span; SOI, surgical orthotopic implantation.

dation by this enzyme and affording a longer plasma half-life (6). Hanaoka et al. (6) has reported potent antitumor activity of CS-682 against a broad spectrum of human tumors in nonmetastasizing s.c.-transplant nude mouse models, including gastric, lung, colon, breast, and ovarian.

For noninvasive detection, differential diagnosis, and staging of benign versus malignant liver tumors, MRI is arguably the most accurate modality currently available in the clinic (high sensitivity and specificity; Refs. 7–9). It allows longitudinally acquired liver assessments to be performed that yield a wealth of information related to anatomical location and underlying physiological change as a function of time and treatment (10, 11). In this study, MRI techniques comparable with those routinely used for the clinical management and assessment of liver disease were performed using an athymic nude mouse model of metastasis. Specifically, T1-weighted MR images routinely yielded superior anatomical detail and shorter MR acquisition times that resulted in reduced motion artifacts (lesions appeared hypointense or darker as compared with adjacent normal tissue; Ref. 12). T2-weighted MR acquisitions yielded improved lesion conspicuity important for characterization and assessment of tumor burden (lesions appear hyperintense or brighter as compared with adjacent normal liver tissue; Refs. 13, 14). Furthermore, to determine the pattern and time course of lesion development in liver, metastatic foci were "visualized" using three-dimensional semitransparent and opaque surface-rendering algorithms.

With these techniques, we report here that treatment with CS-682 demonstrates superior inhibition of the occurrence of metastases than the currently used clinical agents DFDC and 5-FU. The antitumor efficacy of CS-682 resulted in overall improved survival in comparison with the other agents.

MATERIALS AND METHODS

Reagents. CS-682 and DFDC were obtained from Sankyo, Inc. (Tokyo, Japan). 5-FU was purchased from Pharmacia, Inc. (Kalamazoo, MI).

Mice and Tumor Lines. Female athymic nude mice with s.c. implants of AC3488, a highly liver-metastatic tumor derived from a colon cancer patient with liver metastases, were used (2, 3). All of the animals were maintained in an aseptically clean environment. Caging, bedding, food, and water were all autoclaved before use. All of the animal protocols used in this study were approved by Roswell Park Cancer Institute Animal Care and Use Committee (IACUC) and the Department of Laboratory Animal Resources as well as by the IACUC at AntiCancer, Inc., under NIH Assurance no. A3873-1.

Treatment Model. Six groups of 20 nude athymic mice with SOIs of AC3488 on day 0 were used (2, 3, 5–17). Three untreated groups of 20 mice each were included to ascertain that administration of anesthesia and the process of MR image acquisition had no effects on the occurrence of metastasis. These control groups included the following: anesthesia with MRI, anesthesia without MRI, and neither anesthesia nor MRI. Because no significant difference in metastasis was observed (P = 0.52) among these groups, we concluded that any antimetastatic effect seen was unconfounded by the use of anesthesia or by the MRI procedure. The remaining three groups received treatment with CS-682, DFDC, or 5-FU at their respective MTDs (see Deter-
CS-682 INHIBITS LIVER METASTASIS IN A NUDE MOUSE MODEL.

ministration of MTD) beginning on day 13 postimplantation. CS-682 was administered p.o. every day for 5 days/week for 2 weeks (end, day 27); DFDC was administered i.p. every 3 days for 4 doses (end, day 23); and 5-FU was administered i.p. every day for 5 days (end, day 18). At the end of these treatment schedules, mice were observed for long-term survival until death or day 90, when surviving animals were sacrificed by cervical dislocation.

MRI. MRI was begun on day 13 postimplantation of AC3489 and repeated at variable intervals until day 38. Before imaging, mice were allowed to fast for 18–24 h. In preparation for imaging, mice were anesthetized by i.p. administration of ketamine/xylazine (10 mg/ml/1 mg/ml) at a dose of 1.0 ml/100 g. Each animal was secured in a mouse coil chamber and positioned in the scanner. To ensure the same positioning in the subsequent scans, alignment markings noting the center of the liver in each mouse were made.

High resolution MRI scans were performed using a General Electric (GE) CSI 4.7T/33 cm horizontal bore magnet (GE NMR Instruments, Fremont, CA) with upgraded radio-frequency and computer systems incorporating AVANCE digital electronics (Bruker Biospec platform with Paravision Version 2.1 Operating System; Bruker Medical, Billerica, MA). MR data were acquired using a Gd0 removable gradient coil insert generating a maximum field strength of 950 mT/m, a custom-designed 35-mm radio-frequency transceiver coil, standard SE, and RARE SE MRI pulse sequences.

A typical acquisition consisted of a series of scans including a localizer, T1-weighted (or proton-density-weighted) and T2-weighted RARE SE MR images spanning the entire liver and upper abdomen. Coronal images were routinely acquired for each mouse; axial images were periodically obtained in randomly selected mice. Typical acquisition parameters consisted of 1-mm slices with a 2.8 X 4-cm field of view (FOV), 192 X 256 matrix, 4–8 NEX, 20–30 s/scan using TR/TE = 3237/8 ms for T1-weighted SE acquisitions. TR/TE = 4496/41 ms for proton-density-weighted RARE acquisitions and TR/TE = 7725/121 ms for T2-weighted RARE acquisitions. Qualitative assessment of the time to metastatic foci formation, the pattern of metastatic lesions within the liver, and the overall burden of metastatic disease were noted. Mice were imaged at least once in control (14 mice), CS-682 (13 mice), DFDC (15 mice), and 5-FU (14 mice) treatment groups.

Volume Rendering. Image analysis and three-dimensional volume rendering of data was performed using AnalyzePC Version 4.0, (Biomedical Imaging Resource, Mayo Foundation, Rochester, MN). Specifically, raw data from the MR scanner were reformatted and displayed using AnalyzePC. Objects were then created using a combination of auto-segmentation macro based on thresholding and seed-growing algorithms performed on a lesion-by-lesion or tissue-by-tissue basis. Visual inspection of each lesion/tissue using both T1- and T2-weighted data was performed, and results were manually adjusted by tracing individual object boundaries using a digital liquid crystalline display pressure-sensitive graphics tablet (Wacom, PL500 monitor-tablet; Wacom Technologies Corp., Vancouver, WA). Using the AnalyzePC volume rendering tool, we then created transparencies of each object and interactively displayed them in three dimensions. Objects included: normal liver, liver metastasis, kidneys, kidney metastasis, stomach, gall bladder, and major hepatic vessels in liver. From each individual object map, tissue lesion volumes were measured for further analysis. For improved visualization of lesion volume and spatial location within the liver, opaque surface renderings of metastases were also performed.

Histological Analysis. For each mouse, conventional necropsy and H&E histology were performed to ascertain the presence or absence of metastatic foci in the liver. The liver weight of each sacrificed animal was measured at necropsy before histological preparation; liver weights of animals found dead were not obtained.

Table 1. Relative efficacies of CS-682, 5-FU, and DFDC in inhibiting liver metastasis

<table>
<thead>
<tr>
<th>Group</th>
<th>Group size</th>
<th>No. with metastases (% of total group size)</th>
<th>Improvement over control (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-682</td>
<td>20</td>
<td>7 (35%)</td>
<td>61.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DFDC</td>
<td>20</td>
<td>15 (75%)</td>
<td>18.2</td>
<td>0.11</td>
</tr>
<tr>
<td>5-FU</td>
<td>20</td>
<td>12 (60%)</td>
<td>34.5</td>
<td>0.006</td>
</tr>
<tr>
<td>Control</td>
<td>60</td>
<td>55 (91.7%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Determination of MTD. Dose escalations of CS-682, DFDC, and 5-FU were conducted in groups of six athymic nude mice to determine each agent's respective MTD at the given schedules. Preparation of CS-682 involved initial dissolution in 5% dimethylacetamide and additional dilution in 10% Emulphor; CS-682 was administered p.o. daily 5 days/week for 2 weeks, for a total of 10 doses. DFDC was dissolved in 0.9% sodium chloride and was administered i.p. every 3 days for four total doses over 10 days (4). 5-FU was diluted in 0.9% sodium chloride and was administered i.p. daily for 5 days; Ref. 5). An untreated group was also included for comparison purposes.

The maximum tolerated doses of CS-682, DFDC, and 5-FU were defined as the highest dosages at which significant toxicity-related weight loss and toxic death were not observed in athymic nude mice. The MTD of CS-682, administered p.o. on a schedule of every day, 5 days/week, for 2 weeks, was established at 40 mg/kg/dose (Table 1). The MTD of DFDC, administered i.p. on a schedule of every 3 days for 4 doses, was 150 mg/kg/dose. The MTD of 5-FU, administered i.p. on a schedule of every 4 days for 5 doses, was 35 mg/kg/dose. Treatment at these MTDs did not result in significant weight loss or any drug-related death among the mice. In general, drug-related lethality was observed in a significant number of mice only when body weight loss exceeded 20% of the original weight, which occurred at doses of CS-682, DFDC, and 5-FU that exceeded their respective MTDs.

Statistical Analyses. Median time-to-death was estimated for each group of mice via the method of Kaplan and Meier (18). Comparisons between these groups for differences in survival were made using the Cox-Mantel test (19).
The BMDP statistical package was used for these time-to-death analyses. The proportion of mice that developed liver metastases was compared between groups with a standard approach using the MINITAB statistical package. Because 6–8 statistical comparisons were made, a Type I error rate of 0.01, rather than the usual 0.05, was used to compensate for the increased probability of finding a statistically significant difference among the multiple comparisons merely from chance (Bonferroni correction). Therefore, all Ps will be compared with 0.01 when making inferences.

RESULTS

Comparative Efficacy of CS-682, DFDC, and 5-FU Determined by MRI of Onset and Growth of Liver Metastasis. MRI studies were conducted to first ascertain that no liver metastases had been established before the initiation of treatment protocols, which was confirmed in all of the cases before the initiation of treatment. Anatomy of a normal mouse in the coronal plane is shown in Fig. 1a. Major features are labeled including lungs, liver, stomach, spleen, vena cava, kidney, and intestines. A tumor-free liver obtained from a control mouse on day 13 postimplantation is shown in Fig. 1, b and c.

Second, image acquisition and analysis were carried out at various time points between day 13 and day 38 to qualitatively and quantitatively characterize patterns of metastasis among treatment groups. In general, untreated control mice formed metastatic colonies in the liver parenchyma relatively early in the 90-day time course of the experiment. Metastatic tumor colonies progressively increased in number and size over time, often to the point at which the total metastatic burden exceeded 95% of the total liver volume. This is illustrated in Fig. 1, d and e. Results were obtained on day 21 postimplantation from the same mouse depicted in Fig. 1, b and c, and demonstrate tumor involvement of the entire liver.

In contrast, CS-682 treatment appeared to delay the onset of metastatic foci formation among mice in which metastases were eventually observed. In this treatment group, the total burden of metastatic disease for any individual mouse imaged never exceeded 45% of the liver (Fig. 2, a and b). Treatment with DFDC also seemed to substantially delay the formation of metastases relative to control. However, once metastatic lesions were initially established, they were often diffuse in distribution throughout the liver parenchyma, such that they were visually indistinguishable from normal liver tissue. In addition, the volumes of these lesions invariably exceeded 95% of total liver

![MRI results for CS-682, DFDC, and 5-FU-treated mice. MR images were acquired with one of several treatment schedules and agents (see Treatment: Model for details). a, d, g, T1-weighted MR images; b, e, h, T2-weighted MR images; c, f, i, H&E histology sections. a, b, c, representative MR images acquired from animals treated with CS-682. A single, focal metastatic colony is clearly observed. c, H&E histology confirms the focal nature of the lesion. d, e, f, representative images after 5-FU treatment. Multiple focal metastatic liver tumors are apparent as well-circumscribed lesions (arrows). H&E histology confirms the focal nature and relative concentration of the colonies. g, h, i, representative images after DFDC administration. The diffuse, poorly circumscribed hypointense regions on T1-weighted images (g) and hyperintense regions on T2-weighted images (h) depict widespread metastatic disease (arrows) confirmed by H&E histology results (i).](image-url)
volume at the time of detection. This is seen in Fig. 2, c and d. Treatment with 5-FU, in general, did not delay the initial appearance of metastatic lesions, which appear focal rather than diffuse in their pattern of colonization of liver tissue and are shown in Fig. 2, e and f. The overall metastatic burden did increase over time, reaching an observed maximum of 50% of total liver volume. However, tumor growth occurred at a slower rate than was seen in the control group.

**Comparative Efficacy of CS-682, DFDC, and 5-FU Determined by Volume Rendering of MR Liver Metastasis Images.** In an attempt to succinctly summarize spatial relationships observed in this study and to assess lesion colonization relative to major hepatic vessels and liver lobes, semitransparent three-dimensional renderings from each of the treatment groups were performed. The first column in Fig. 3, a, c, e, and g, shows anterior to posterior renderings of liver, metastases, and nearby structures visualized at 0° rotation for improved spatial localization. The following opacities and pseudocolor image maps were used: (a) liver metastasis, red with opacity = 0.7; (b) normal liver, cyan with opacity = 0.25; (c) kidneys, green with opacity = 0.5; (d) stomach, yellow with opacity = 0.5; (e) gall bladder, white with opacity = 0.7; and (f) major hepatic vessels, purple with opacity = 0.9. Fig. 3e is shown with an opacity = 0.1 for liver metastases and an opacity = 0.5 for normal liver because normal liver tissue was observed interior to metastases. The second column in Fig. 3, b, d, f, and h) shows three-dimensional opaque surface renderings of metastases without normal liver and nearby structures for improved visualization of lesion morphology.

Representative renderings from the CS-682-treated group obtained on day 36 post-SOI (Fig. 3, a and b) reveals the focal nature of metastatic lesions and the relative paucity of lesions compared with other treatment groups, including untreated control. Representative results from the 5-FU-treated group obtained on day 24 post-SOI are shown in Fig. 3, c and d. Although metastases are focal, as was seen among the CS-682-treated animals, they are more numerous and larger in this group.

In contrast, representative renderings from the DFDC-treated group (day 24 post-SOI) and the untreated control group (day 27 post-SOI) in Fig. 3, e and f, and Fig. 3, g and h, respectively, reveal extensive spatial distributions of metastases as well as an overall enlargement of

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**Fig. 3.** Three-dimensional renderings for spatial localization of liver metastasis. *First column*, a, c, e, g, anterior-to-posterior semitransparent renderings of liver metastases and relevant nearby structures. *Second column*, b, d, f, h, opaque surface renderings of metastatic lesions for improved visualization of their relative volume and spatial location within the liver. a, b, results obtained after CS-682. Notable are the focal nature and relative decrease in lesion occurrence as compared with 5-FU, DFDC, and control groups. c, d, results obtained after 5-FU. e, f, results obtained after DFDC treatment; liver is enlarged, and metastases are extensive and heterogeneous. g, h, representative results for untreated control mice. See Volume Renderings for details.
total liver volumes. In the DFDC-treated group, a diffuse pattern of metastases without discernable foci is apparent, whereas the margins of some lesions in the control group reveal foci formation against a diffuse background of metastases.

**Comparative Efficacy of CS-682, DFDC, and 5-FU on Liver Metastasis Determined by Histology.** Relative to DFDC, 5-FU, and control groups, CS-682 demonstrated a superior inhibition of metastasis from the primary tumor site in the cecum to the liver (Table 1). Untreated mice exhibited a 91.7% frequency of metastasis, as determined by necropsy at the time of death or on termination of the time course at day 90. At a metastasis rate of 35%, mice receiving CS-682 exhibited 61.8% fewer metastases than did untreated control mice ($P < 0.001$). At a metastasis rate of 75%, mice treated with DFDC exhibited an insignificant 18.2% fewer metastases than did untreated control mice ($P = 0.11$). Treatment with 5-FU resulted in a 60% frequency of metastasis, which represents 34.5% fewer metastases than in untreated controls ($P = 0.006$).

Liver weights were obtained as proxy measures of tumor burden in the liver (Table 2). At 2.1 g and 3.1 g, the median liver weights of animals treated with CS-682 and 5-FU, respectively, were significantly lower than median liver weights of controls, suggesting a decreased relative burden of metastases in the liver. In comparison, DFDC-treated animals demonstrated similar median weights as in controls, suggestive of comparable tumor burdens.

**Comparative Efficacy of CS-682, DFDC, and 5-FU on Survival.** Treatment with CS-682 improved survival in mice after the cessation of treatment to a greater extent than in DFDC, 5-FU, and control groups (Fig. 4; Table 3). ILS with CS-682 treatment was 28 days beyond the 29-day mean survival without treatment, which represents a 96.6% improvement over control ($P < 0.0001$). The ILS was 11 days (37.9%) with DFDC treatment ($P = 0.0105$). The ILS was 14 days (48.3%) with 5-FU treatment ($P = 0.0010$).

**Table 2** Liver weights of CS-682, 5-FU, and DFDC

<table>
<thead>
<tr>
<th>Group</th>
<th>Group size</th>
<th>Median liver weight (g)</th>
<th>Weight difference relative to control (g)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-682</td>
<td>17</td>
<td>2.1</td>
<td>-2.9</td>
<td>0.038</td>
</tr>
<tr>
<td>DFDC</td>
<td>14</td>
<td>5.1</td>
<td>+0.1</td>
<td>0.76</td>
</tr>
<tr>
<td>5-FU</td>
<td>17</td>
<td>3.1</td>
<td>-1.9</td>
<td>0.0042</td>
</tr>
<tr>
<td>Control</td>
<td>59</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3** Survival efficacy of CS-682, 5-FU, and DFDC

Each group consisted of 20 mice. Each agent was administered at its MTD. All of the mice surviving to day 90 were sacrificed. Improvement over control reflects the ILS of treatment groups relative to the median survival of 29 days among the control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Group size</th>
<th>Median day of death (range)</th>
<th>Improvement over control (%)</th>
<th>$P$</th>
<th>No. of long-term survivors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-682</td>
<td>20</td>
<td>57 (17-90+)</td>
<td>96.6</td>
<td>&lt;0.0001</td>
<td>9 (45)</td>
</tr>
<tr>
<td>DFDC</td>
<td>20</td>
<td>40 (22-90+)</td>
<td>37.9</td>
<td>0.0105</td>
<td>2 (10)</td>
</tr>
<tr>
<td>5-FU</td>
<td>20</td>
<td>43 (22-90+)</td>
<td>48.3</td>
<td>0.0010</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Control</td>
<td>60</td>
<td>29 (20-70)</td>
<td></td>
<td></td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Long-term survival was defined as the overall number of animals that survived to the termination of the experiment at day 90. With 45% of mice surviving to day 90, the CS-682 treatment group demonstrated the most long-term survivors of all of the groups compared, followed by the 5-FU treatment group (25%) and the DFDC treatment group (10%). The untreated control group had no animals that survived to the termination of the experiment (Table 3).

**DISCUSSION**

Observations made from MRI data clearly demonstrate the efficacy of CS-682 as an antimetastatic agent. MRI also provides further insight into the metastatic process. MRI showed that mice treated with CS-682 remained free of liver metastases for much longer than did control and 5-FU-treated groups. CS-682-treated mice also exhibited a decreased burden of liver metastases, as demonstrated by MR-derived volumetric calculations of foci, relative to the other groups. Although MR images indicated that DFDC treatment delayed the formation of metastatic foci initially, this group eventually demonstrated the highest percentage of mice developing metastases among the three treatment groups. This suggests that DFDC may be effective in initially delaying the onset of foci formation, but the inhibition is transient. Widespread diffuse metastases subsequently form such that the long-term outcome is not improved by DFDC therapy.

Three-dimensional visualization of the MR data sets was undertaken because spatial localization of liver metastasis often plays a significant role in clinical treatment-planning strategies, especially if surgery is one option being considered. No preferential metastatic sites within specific liver lobes or regions was consistently observed. However, three-dimensional visualization of metastatic spread demonstrated that CS-682 treatment consistently resulted in a reduced number of metastases that were generally more focal in nature and represented a lower metastatic-volume:total-liver-volume ratio than did control or other treatment.

The difference in liver metastasis incidence seen between CS-682 and DFDC treatment groups (61.8 versus 18.2% inhibition of liver metastasis), as determined by histological methods, represents a significant difference ($P = 0.005$), which indicates that CS-682 is clearly superior to DFDC. The superiority of CS-682 in inhibiting the occurrence of metastasis correlates with the improved survival in CS-682-treated mice compared with DFDC- and 5-FU-treated mice.

Previous studies have demonstrated the antitumor efficacy of CS-682 in both in vitro and in nonmetastatic s.c. in vivo models of cancer. In the present report, we demonstrate that CS-682 is also a potent inhibitor of liver metastasis. In conclusion, we report the superior antimitastatic efficacy of CS-682, relative to the commonly used clinical agents 5-FU and DFDC, in a mouse model of liver metastasis. Thus, we propose that CS-682 warrants further evaluation for potential utility as a clinical therapeutic agent for liver metastasis.

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