Tumor-Targeted *Salmonella* Expressing Cytosine Deaminase as an Anticancer Agent

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ABSTRACT

The study was designed to evaluate whether TAPET-CD, an attenuated strain of *Salmonella typhimurium* expressing *Escherichia coli* cytosine deaminase (CD), was capable of converting nontoxic 5-fluorocytosine (5-FC) to the active antitumor agent 5-fluorouracil (5-FU). The antitumor effect of TAPET-CD plus 5-FC against subcutaneously implanted colon tumors was also evaluated. TAPET-CD was given to tumor-bearing mice by a single bolus intravenous administration followed with 5-FC by intraperitoneal administration. TAPET-CD accumulated in tumors at levels 1000-fold higher than that in normal tissues and high levels of 5-FU were detected in tumors in mice treated with both TAPET-CD and 5-FC. No 5-FU could be detected in normal tissues. Inhibition of tumor growth was observed in mice treated with either TAPET-CD alone or TAPET-CD in combination with 5-FC (TAPET-CD/5-FC), but not with 5-FC alone. TAPET-CD/5-FC inhibited tumor growth by 88%–96%, compared to TAPET-CD alone, which inhibited tumor growth by 38%–79%. These data suggest that tumor-targeting *Salmonella* could be used to deliver prodrug-converting enzyme selectively to tumors and produced anti-tumor effects when the corresponding prodrug was also given.

OVERVIEW SUMMARY

TAPET-CD, injected intravenously to tumor-bearing mice, accumulates preferentially in tumors compared to normal tissues. TAPET-CD also converts 5-fluorocytosine (5-FC) to agent 5-fluorouracil (5-FU) selectively in tumors in mice treated with TAPET-CD and 5-FC. No 5-FU can be detected in normal tissues. The combination of TAPET-CD and 5-FC effectively suppresses the growth of murine C38 colon carcinoma and two human colon tumor xenografts, LoVo and WiDr. TAPET-CD plus 5-FC is relatively nontoxic in mice and monkeys at doses that produce therapeutic effects. These studies demonstrate the potential use of attenuated *Salmonella* as a tumor-selective protein delivery vector.

INTRODUCTION

THE PRIMARY LIMITATION of cancer therapy is the lack of selectivity of therapeutic agents to cancer cells. Because of this lack of selectivity, anticancer agents elicit toxicity against normal tissues, which ultimately limits the doses that can be given to cancer patients. Many current discovery and development efforts are devoted to finding anticancer agents that selectively target cancer cells to improve the therapeutic index. An alternative approach is to restrict the distribution of drugs to tumors using local-regional administration methods. In order to increase the exposure of drugs to tumors, techniques such as tumor-specific antibodies also have been used to localize cytotoxic agents in tumor cells. An attenuated strain of *Salmonella typhimurium* (VNP20009; Vion Pharmaceuticals, Inc., New Haven, CT) has recently been developed. The attenuation is partly caused by the disruption of the *msbB* gene (Low *et al.*, 1999b), which regulates the addition of a terminal myristoyl group to lipid A. Lipopolysaccharide isolated from *Salmonella* expressing the mutated lipid A show a markedly diminished ability to induce tumor necrosis factor-α (TNF-α). The attenuation is also due to the deletion of the *purI* gene, causing VNP20009 to require an external purine source for survival (Low *et al.*, 1999a).

We have previously shown that VNP20009 could be a good vector for tumor-selective delivery of protein-based antitumor agents. VNP20009 is genetically stable, as demonstrated *in vivo* and *in vitro* (Clairmont *et al.*, 2000). Compared to wild-type *Salmonella*, the pathogenicity or toxicity of VNP20009 is reduced or eliminated more than 10,000-fold. Phase 1 studies in cancer patients (Toso *et al.*, 2002) and studies of several animal species (Lee *et al.*, 2000) demonstrate a good safety profile for VNP20009. According to animal studies (Zheng *et al.*, 2000), VNP20009 preferentially accumulates in tumors (with tumor to normal tissue ratios of 300–25,000:1), and it persists in tumor tissue for more than 4 weeks. VNP20009 exhibits antitumor properties, with tumor size reduction lasting for up to 50–60 days after a single intravenous administration in a variety of murine models (Luo *et al.*, 2002). Because VNP2009 is sensitive to a number of antibiotics, it can be conveniently eliminated from the body. In contrast, other vector systems such as viruses or liposomes cannot be eliminated easily from the body. In addition, VNP20009 is environmentally friendly because it has difficulty surviving freely in the environment.

Using cytosine deaminase (CD) and green fluorescent protein genes as markers, we further demonstrated that VNP20009 could be used as a tumor-selective vector for delivering antitumor agents (Zheng *et al.*, 2000). TAPET-CD is generated by the incorporation of the CD gene from *Escherichia coli* into VNP20009. CD is an enzyme found in bacteria and fungi, but not in mammalian cells. The enzyme converts 5-fluorocytosine (5-FC), a relatively nontoxic agent, to the cytotoxic antimetabolite 5-fluorouridine (5-FU) (Deonarain *et al.*, 1995). The activated agent is then converted to 5'fluorouridine-5'triphosphate and 5-fluoro-2'deoxyuridine-5' monophosphate, resulting in the disruption of RNA and DNA synthesis with subsequent toxicity to both quiescent and proliferating cells. 5-FU is currently used in the clinical treatment of colorectal, stomach, head and neck, and breast carcinomas. Although 5-FU is not as toxic as some antitumor alkylating agents, sufficiently high levels of 5-FU have been achieved *in vivo* that are capable of eradicating tumors after retroviral transduction of the CD gene, even when CD incorporates into only 2% of tumor cells (Huber *et al.*, 1994). Cumulative clinical data suggest that the antitumor activity of 5-FU may be directly related to the duration of exposure and its concentration within the tumor. One approach for achieving high and prolonged tumor concentrations of 5-FU involves coadministration of TAPET-CD and 5-FC. TAPET-CD maintains its VNP20009 properties (Lee *et al.*, 2001) and selectively produces high levels of CD in tumors compared to normal tissues (Zheng *et al.*, 2000). We report here that TAPET-CD/5-FC induces prolonged, high concentrations of 5-FU in tumor tissues, causing a reduction in tumor size greater than that induced by TAPET-CD or 5-FC alone.

MATERIALS AND METHODS

TAPET-CD genetic manipulation

The CD gene was isolated from *E. coli* using procedures described by Laliberte and Momparler (1994) CD was initially cloned into pTrxFus (In Vitrogen, Carlsbad, CA) to encode a thioredoxin-CD fusion protein, then the fusion protein gene was subcloned into pTrc99A. The cloned DNA was transformed into *Salmonella* as described (Pawelek *et al.*, 1997).

Cell culture

Murine B16-F10 melanoma cells were obtained from Dr. I. Fidler (M.D. Anderson Cancer Center, Houston, TX). C38 colon carcinoma cells were obtained from NCI (Frederick, MD). Widr and LoVo colon carcinoma xenografts were obtained from the American Type Culture Collection (Rockville, MD). Cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. At approximately 80% confluence, cells were detached from the flasks by addition of 2 ml of trypsin, resuspended in 25 ml phosphate-buffered saline (PBS), and transferred into a 50-ml Falcon conical centrifuge tube. Cells were pelleted by centrifugation at 4°C for 5 min at 800 revolutions per minute (rpm) in a Beckman (Fullerton, CA) GS-6R refrigerated centrifuge. The supernatant was discarded, and the cell pellet was resuspended in PBS. The tumor cell suspension was kept on ice until implantation into mice.

Animals

Female C57BL/6 and *nu/nu* CD-1 female mice were obtained from Charles River Laboratories (Wilmington, MA). Animals used in the studies were as uniform in age and weight as possible. They were approximately 8 weeks of age, and body weights of C57BL/6 mice and *nu/nu* CD-1 mice ranged from 19–21 g and 23–26 g, respectively. All animals were kept in a well-ventilated room in which a 12-hr light/12-hr dark photoperiod was maintained. Room temperature was maintained between 72°F ± 2°F.

Quantitation of TAPET-CD accumulation and 5FC/5FC conversion in tissues

Animals were inoculated intravenously with 1×10^6 colony-forming units (cfu) of TAPET-CD on day 14 after B16-F10 melanoma tumor implantation. Mice received 300 mg/kg of 5-FC intraperitoneally on day 17, 3 days after TAPET-CD injection. They were killed by inhalation of CO_2 at 10, 30, 90, 270, and 360 min after 5-FC injection. Tissues, including tumor, liver, spleen, brain, whole blood, and bone marrow were weighed and homogenized in PBS, and bacteria were quantitated by plating serial dilutions of the homogenates onto msbB plates, incubating overnight at 37°C, and counting bacterial colonies. The conversion of 5-FC to 5-FU in tissues including tumor, liver, spleen, brain, serum, and bone marrow, was determined by high-performance liquid chromatography (HPLC) analysis. The detection limit for 5-FC and 5-FU was 0.5 $\mu g/g$ and 0.2 $\mu g/g$, respectively.

Antitumor activity of TAPET-CD

For the murine tumor model, C38 tumor tissue was aseptically dissected from a tumor-bearing C57BL/6 mouse. The tumor was mechanically minced into 3–5 mm^3 pieces, and transplanted subcutaneously with a 16-gauge trocar needle into the right flank of C57BL/6 mice under methoxyflurane anesthesia. Fifteen days after transplantation, when C38 tumors had grown to a volume of approximately 300 mm^3, the mice were ran-

domized and divided into 4 groups of 10 animals each. Groups 1 and 3 received PBS (0.1 ml). Groups 2 and 4 were injected in the tail vein with TAPET-CD at 1×10^6 cfu per mouse on day 15. Groups 3 and 4 received daily three intraperitoneal injections of 5-FC at a dose of 300 mg/kg on days 19 through 40.

WiDr cells were implanted subcutaneously (5×10^6 cells) into *nu/nu* CD-1 mice (day 0). By day 21, mice had similar tumor sizes of approximately 250 mm^3 as determined by electronic caliper measurements. They were randomly assigned into 4 groups of 7–8 animals each. Groups 1 and 3 received PBS (0.1 ml). Groups 2 and 4 were injected in the tail vein with TAPET-CD at 1×10^6 cfu per mouse. Groups 3 and 4 received daily three intraperitoneal injections of 5-FC at a dose of 300 mg/kg on days 23 through 44.

LoVo human colon carcinoma cells were implanted subcutaneously into *nu/nu* CD-1 mice. By day 20, tumors had grown to approximately 150 mm^3. Groups 1 and 3 received PBS (0.1 ml). Groups 2 and 4 received a single dose of TAPET-CD at 1×10^6 cfu per mouse through the tail vein. Groups 3 and 4 received daily three intraperitoneal injections of 300 mg/kg 5-FC on days 26 through day 53.

In another control experiment, CD-1 nude mice were implanted with Widr human colon tumors and treated with 5-FU, either alone or in combination with TAPET-CD when tumor reached approximately 250 mm^3. 5-FU was given intraperitoneally to mice 5 days after TAPET-CD administration, once daily for 5 days at 50 mg/kg per day.

Tumor volume was measured in three dimensions twice weekly and calculated with the formula $L \times H \times W/2$, where L, H, and W represent length, height, and width, respectively. Tumor volume was presented as mean $\pm$ standard deviation and Student's *t* test was performed for statistical analysis.

RESULTS

TAPET-CD tissue distribution

The distribution of TAPET-CD was determined by quantitating the amount of TAPET-CD in tumors and normal tissues. Three days after TAPET-CD injection, the highest levels of TAPET-CD occurred in tumors ($1.1 \times 10^9 \pm 1.4 \times 10^8$ cfu/g), followed by spleen ($9.3 \times 10^6 \pm 3.1 \times 10^5$ cfu/g) and liver ($2.2 \times 10^5 \pm 8.0 \times 10^4$ cfu/g). The amount of TAPET-CD in brain, bone marrow, and whole blood was approximately four to five orders of magnitude lower than that found in tumors (Fig. 1). These results indicate that TAPET-CD preferentially accumulates and proliferates in tumors rather than in normal tissues. Tissue distribution of TAPET-CD in non-tumor–bearing mice was similar except that approximately 10 times less bacteria were detected in the brain (data not shown).

In Vivo 5-FC/5-FU conversion in tumors by TAPET-CD

The concentrations of 5-FC in serum and different tissues are shown in Figure 2A. The concentration of 5-FC in the untreated control group was undetectable (data not shown). Similarly, 5-FC could not be detected in mice treated with 60 mg/kg 5-FU. In mice treated with 5-FC only, the average concentration of 5-FC was 1675 μg/ml in serum, 260 μg/g in liver, 119

μg/g in tumor, 103 μg/g in spleen, 57 μg/g in bone marrow, and 13 μg/g in brain at 30 min after administration of 300 mg/kg 5-FC. In mice previously treated with TAPET-CD, the tissue distribution of 5-FC in the above tissues was similar to those of mice treated with 5-FC alone, indicating that TAPET-CD treatment may not change the distribution pattern of 5-FC in the body. The concentrations of 5-FU in serum and different tissues in mice receiving 5-FC, with and without TAPET-CD, were also determined. As shown in Figure 2B, no 5-FU was detected in any tissues of the group treated with 5-FC alone, indicating that no endogenous conversion of 5-FC to 5-FU occurs in the body. In the group treated with 5-FU alone, mice received a single therapeutic dose of 60 mg/kg. The concentrations of 5-FU in the evaluated tissues ranged from 0.7 to 10 μg/g at 30 min after 5-FU injection. The average level of 5-FU in tumors (4 μg/g) was significantly lower than that in serum (10 μg/ml) and liver (6 μg/g) and similar to that in bone marrow (5 μg/g) and spleen (4 μg/g). However, the highest levels of 5-FU were detected in tumor tissues of mice receiving both TAPET-CD and a single dose of 300 mg/kg of 5-FC. The 5-FU levels reached 19 μg/g in tumors 30 min after 5-FC administration, 4.75-fold more than that found in tumors of mice treated with 60 mg/kg of 5-FU. No 5-FU was detected in other tissues in mice receiving TAPET-CD/5-FC. These results indicate that 5-FC was efficiently converted to 5-FU in tumors by CD, which was expressed by high levels of accumulated TAPET-CD. Because TAPET-CD in other tissues was about 1000-fold lower than in tumors, there was insufficient CD in these tissues to convert 5-FC to 5-FU.

Pharmacokinetics of 5-FC and converted 5-FU in tissues

The pharmacokinetics of 5-FC conversion to 5-FU in tumors are shown in Figures 3A and 3B. After a single intraperitoneal injection of 5-FC (300 mg/kg), 5-FC in tumors quickly reached a level of 160 μg/g at 10 min, then gradually declined and became undetectable at 90 min. The comparative 5-FC levels in the tested tissues were: Serum > liver > tumor > spleen > bone marrow > brain. Peak 5-FC concentrations ranged from 15 μg/g in the brain to 2,000 μg/ml in serum.

The pharmacokinetic pattern of converted 5-FU in tumors was similar to that of 5-FC in mice treated with TAPET-CD/5-FC. The concentration of converted 5-FU reached a peak level of 19 μg/g at 30 min in tumors and declined gradually to 5 μg/g at 270 min after 5-FC injection. Low levels of 5-FU (< 0.2 μg/g) were occasionally detected in a few samples in bone marrow and spleen at one or two time points. An intraperitoneal dose of 5-FU (60 mg/kg) gave an intratumoral peak level of approximately 4.7 μg/g at 30 min after administration.

Antitumor activity of TAPET-CD

Figure 4A shows the antitumor activity of TAPET-CD/5-FC in C38 murine colon tumors. Tumor growth of C38 was not altered by treatment with 5-FC. A significant inhibition (79% at day 40) of tumor growth was obtained in mice that received a single injection of TAPET-CD. In the group treated with TAPET-CD/5-FC, all tumors underwent regression during the first week of treatment, reached their nadir (96% inhibition) on day 26, and then grew slowly, reaching approximately their
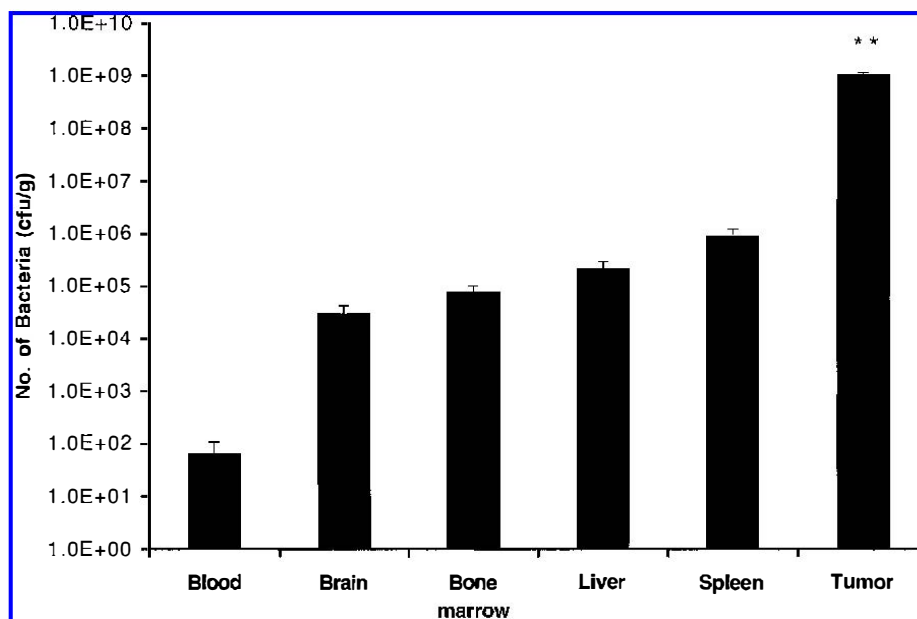
FIG. 1. Tissue distribution of TAPET-CD in mice implanted with B16-F10 melanoma. Mice were administered intravenously with TAPET-CD at 1×10^6 colony-forming units (cfu) per mouse 14 days after tumor implantation. Bacteria content in tissues were evaluated 3 days after bacteria inoculation. **denotes $p < 0.01$ between tumor and normal tissues.

original sizes on day 40. The tumors in the TAPET-CD/5-FC–treated animals were significantly smaller than those in either the 5-FC or TAPET-CD groups from day 26 to the completion of the experiment ($p < 0.01$ between TAPET-CD/5-FC and TAPET-CD on day 40).

As indicated in Figure 4B, WiDr tumors in the PBS control group grew exponentially, increasing in size 10- to 12-fold over the time period examined. 5-FC had little effect on the growth of WiDr tumors in mice (12.5% inhibition on day 47). A single injection of TAPET-CD caused some degree of tumor inhibition (58%) on day 47. The antitumor effect of TAPET-CD/5-FC was not apparent until after 1 week of 5-FC injection. At the completion of the experiment (day 47), profound antitumor effects (88% inhibition) were observed in the group treated with TAPET-CD/5-FC. $p < 0.05$ was obtained for TAPET-CD/5-FC versus TAPET-CD on day 47.

TAPET-CD alone had only modest antitumor effects (38% inhibition at day 54) in LoVo tumors. Pronounced antitumor activity was obtained in mice treated with TAPET-CD/5-FC. The treatment of TAPET-CD/5-FC resulted in an 89% inhibition of tumor growth compared to the control group. No effects on tumor growth were observed in the control group treated with 5-FC alone (Fig. 4C). $p < 0.01$ was obtained for TAPET-CD/5-FC versus TAPET-CD on day 54.

Similar weight loss (10%–15% of original body weight) was observed in mice treated with TAPET-CD alone and TAPET-CD/5-FC. At 300 mg/kg, 5-FC was relatively nontoxic to mice. No animals died in groups treated with TAPET-CD alone or TAPET-CD/5-FC.

5-FU, at 50 mg/kg, given once daily for 5 days, produced significant toxicity when used alone or in combination with TAPET-CD. Weight loss in mice treated with 5-FU was greater than 15% of original body weight. The experiment was terminated 10 days after 5-FU dosing because of severe toxicity. The

mortality in both groups treated with 5-FU and TAPET-CD/5-FU was greater than 40%. No additive antitumor activity between 5-FU and TAPET-CD could be observed because of the early termination of the experiment.

DISCUSSION

We have previously demonstrated that the genetically modified *Salmonella typhimurium*, VNP20009, targets and replicates intratumorally in syngeneic tumors and human tumor xenografts in murine models (Pawelek *et al.*, 1997; Zheng *et al.*, 2000). The use of VNP20009 as a delivery system for antitumor agents has numerous advantages for gene therapy. First, amplification within tumors is highly selective, surpassing the specificity observed for tumor-specific antibodies. Second, amplification theoretically requires only one bacterium to seed the tumor site, making delivery highly efficient and increasing the likelihood of reaching metastases that are inaccessible to other therapeutic methods. Third, systemic administration enables accumulation of the therapeutic vector at tumor sites. Fourth, the expression of enzymes or proteins at high levels makes the vector ideal for carrying prodrug-converting enzymes, cytokines, antiangiogenic peptides, antigenic peptides for immunomodulation, or other agents individually or in combination into tumors. Fifth, long-term, sustained expression of antitumor agents within a tumor can be achieved, because of the slow clearance of bacteria from the tumor.

In this study, we have evaluated the tissue distribution of TAPET-CD, *in vivo* CD activity by measuring the conversion of 5-FC to 5-FU, and antitumor activity of TAPET-CD, both alone and with 5-FC. We demonstrated that TAPET-CD preferentially replicates and accumulates in tumors and consistently expresses active CD that converts 5-FC to 5-FU efficiently and
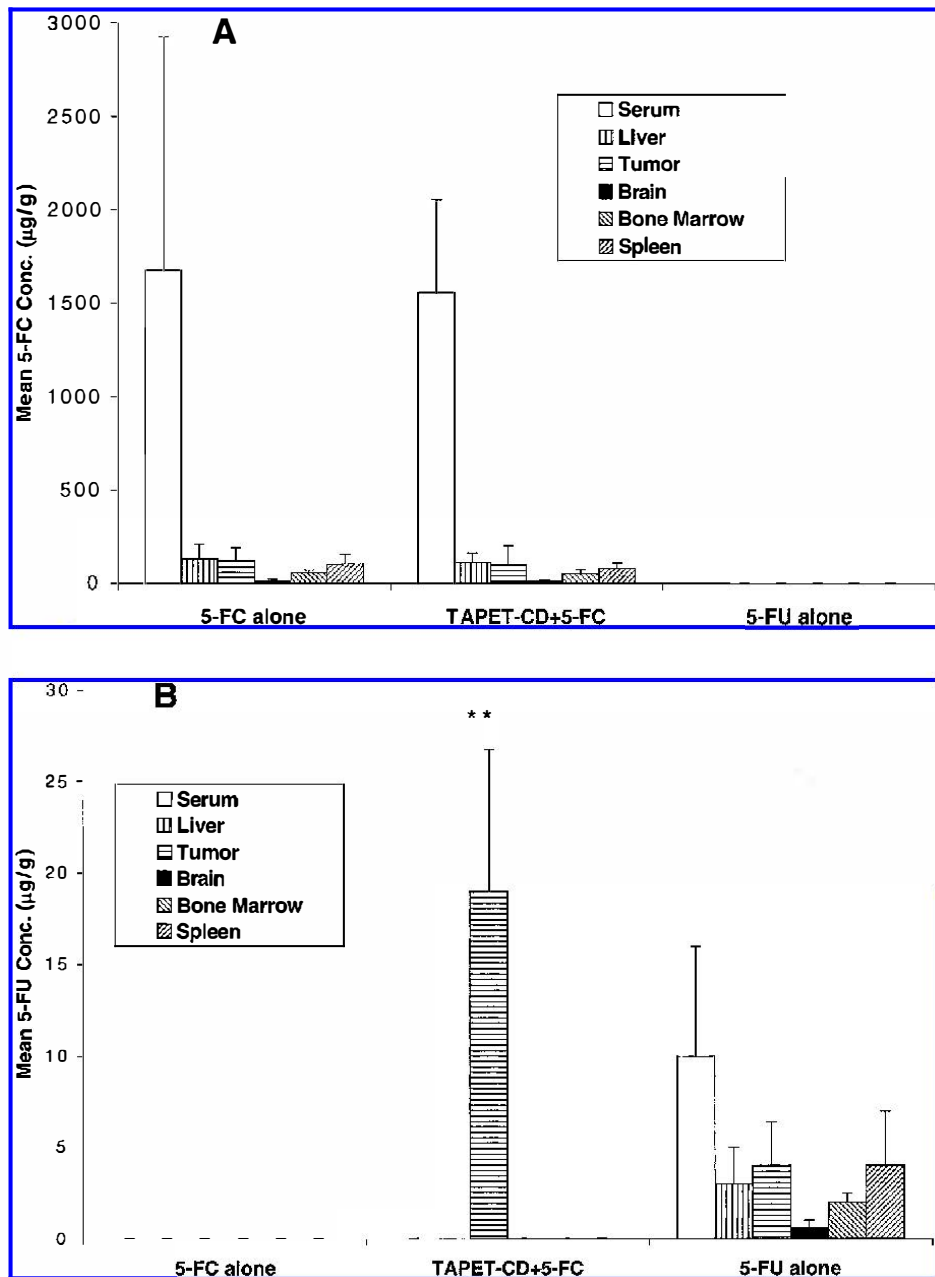
FIG. 2. Tissue distribution of 5-fluorocytosine (5-FC) (**A**) and 5-fluorouracil (5-FU) (**B**) in mice implanted with B16-F10 melanoma. Mice were administered intravenously with TAPET-CD at 1×10^6 colony-forming units (cfu) per mouse 14 days after tumor implantation. Tissues were collected 30 min after a single dose of 5-FC (300 mg/kg) or 5-FU (60 mg/kg) was administered intraperitoneally. For 5-FC and 5-FU groups, phosphate-bufferal saline (PBS) instead of TAPET-CD was given. Three mice were used for each group and **denotes $p < 0.01$ between groups treated with 5-FU alone and TAPET-CD/5-FC.

locally. Because the amount of TAPET-CD in normal tissues is approximately 1000-fold less than that in tumors, the conversion of 5-FU in normal tissues was undetectable. After a single dose of 300 mg/kg 5-FC, the converted 5-FU in tumors reached the highest level (19 μg/g) at 30 min, then gradually declined at 4.5 hr to 5 μg/g, a level that remains cytotoxic to many tumor cell lines. A minimal or undetectable level of 5-FU was found in normal tissues. In contrast, when given directly to mice, 5-FU distributed relatively randomly in most tissues, with levels of 5-FU in blood, liver, and spleen becoming

higher than in tumors. For example, at 60 mg/kg 5-FU, a dose that causes mild toxicity, intratumoral concentrations of 5-FU reached 4.7 μg/g at 30 min and dropped below an effective concentration of 1 μg/g at 90 min. In a cancer patient receiving 300 mg of the antitumor drugs UTF or tegafur, which are derivatives of 5-FU, intratumoral concentrations of 5-FU were approximately 0.18 μg/g at 5.5 hr after drug administration (Arima *et al.*, 1986). TAPET-CD/5-FC clearly produced a higher and more prolonged presence of 5-FU in tumors. Higher intratumoral 5-FU levels could likely produce a higher antitu-
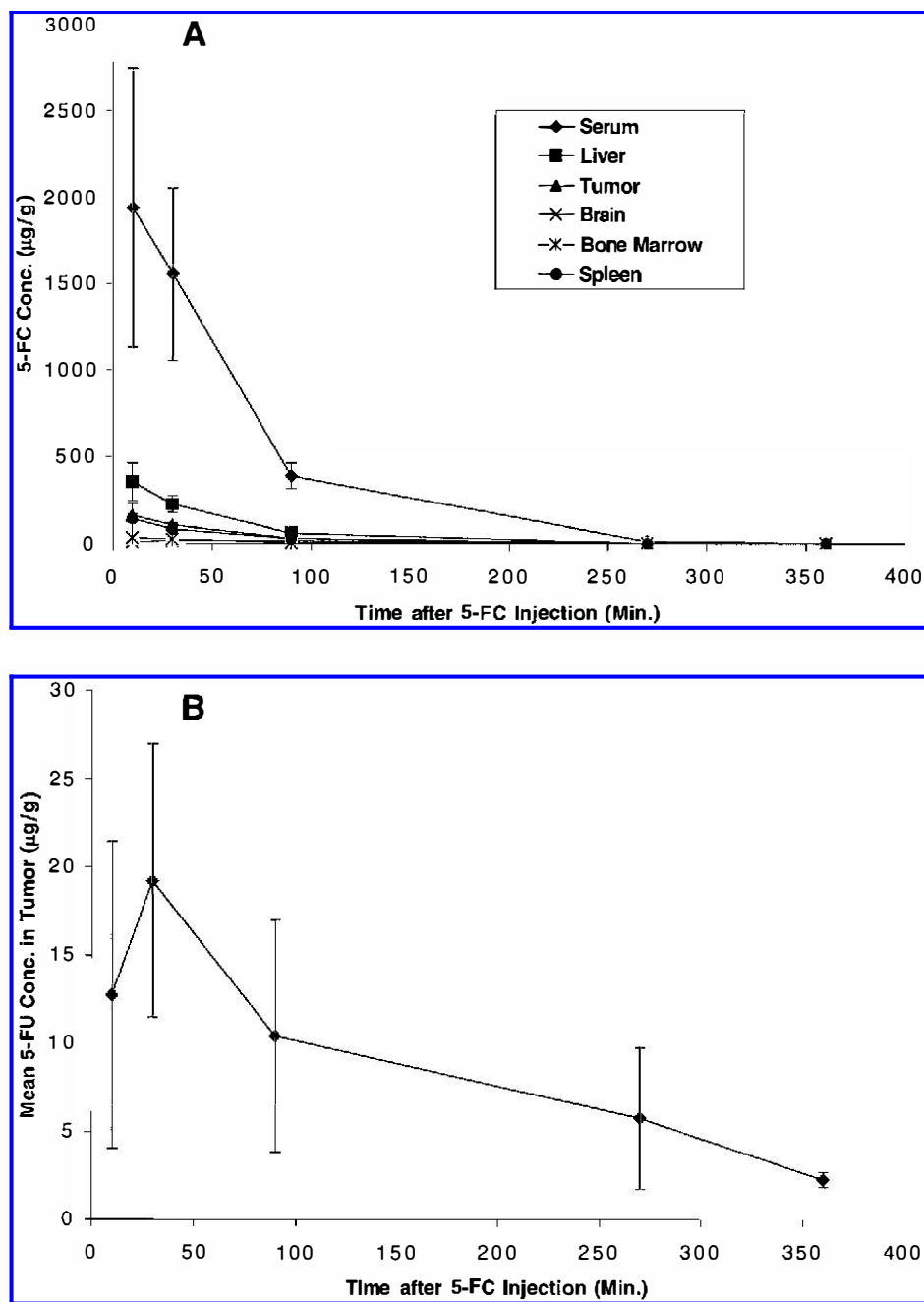
FIG. 3. Pharmacokinetics and tissue distribution of 5-fluorocytosine(5-FC) (**A**) and 5-fluorouracil(5-FU) (**B**) in mice implanted with B16-F10 melanoma. Mice received TAPET-CD intravenously at 1×10^6 colony-forming units (cfu) per mouse 14 days after tumor implantation. Tissues were collected 10 to 360 min after a single dose of 5-FC (300 mg/kg) or 5-FU (60 mg/kg) was administered intraperitoneally to TAPET-CD group or phosphate-buffered saline (PBS)-treated group, respectively. Three mice were used for each group.

mor effect (Presant *et al.*, 1994; Gamelin *et al.*, 1996; Muller *et al.*, 2000).

Using murine and human colon tumor models, we unequivocally demonstrated that TAPET-CD/5-FC produced a superior

antitumor effect than either agent acting alone. Tumor regression was observed in some mice receiving the combination regimen. TAPET-CD and CD activity persisted in B16-F10 melanomas for a minimum of 14 days (Zheng *et al.*, 2000). During

FIG. 4. Tumor growth inhibition of C38 murine colon carcinoma (**A**), WiDr human colon carcinoma (**B**), and LoVo human colon carcinoma (**C**) by TAPET-CD and 5-flurocytosine (5-FC). TAPET-CD [1×10^6 colony-forming units (cfu) per mouse] was injected intravenously to mice bearing C38 (day 15), WiDr (day 21), or LoVo (day 20) carcinoma when tumor reached 150–300 mm^3. 5-FC was given intraperitoneally at 300 mg/kg, three times daily, as indicated. Seven to 10 animals were used per group. *denotes $p < 0.05$ and **denotes $p < 0.01$ between TAPET-CD alone and TAPET-CD/5-FC treated group.
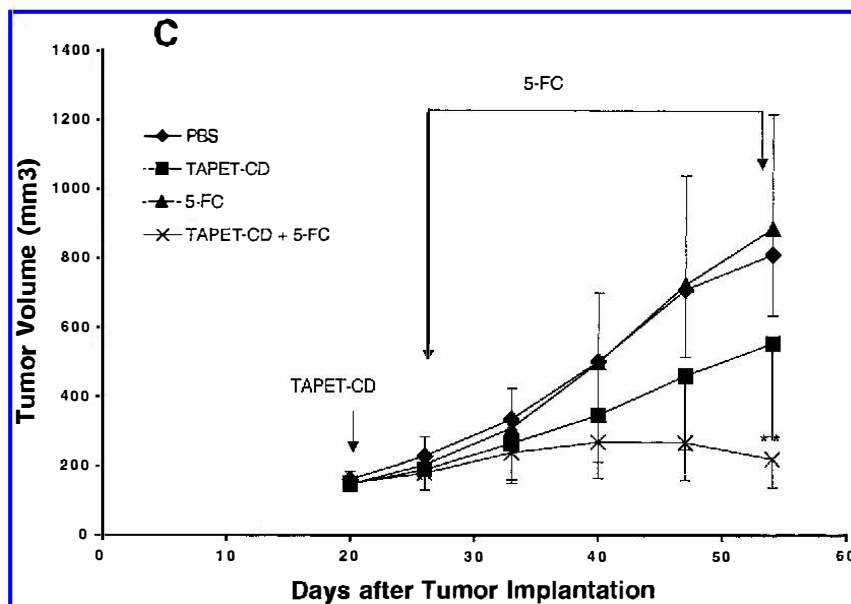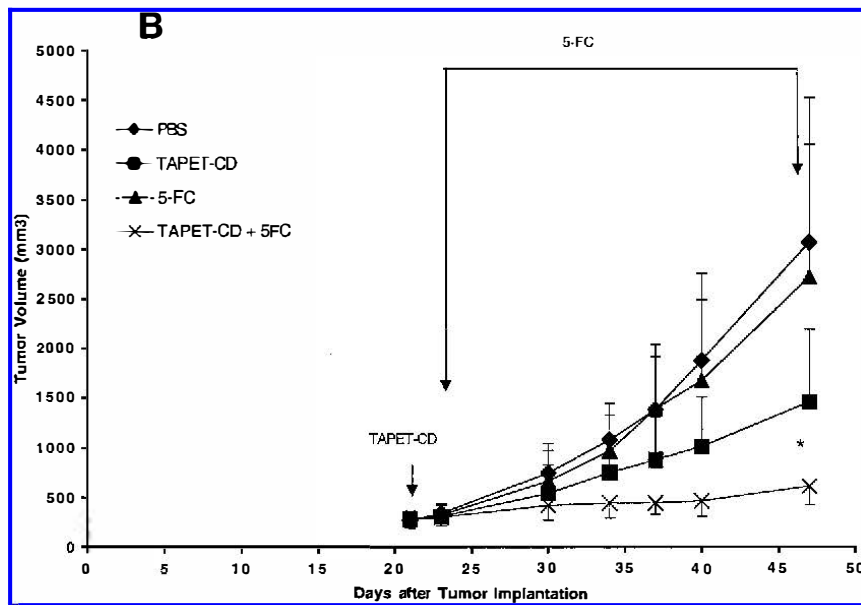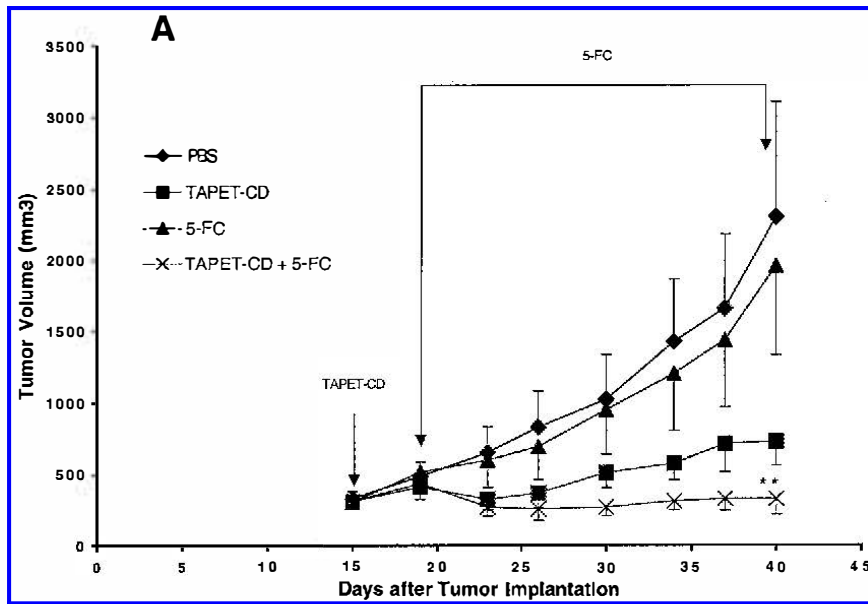
the course of the *in vivo* efficacy experiment, we further detected the presence of TAPET-CD in LoVo colon tumors for up to 32 days after bacteria administration (data not shown). It is clear that a single injection will provide CD for an extended period of time. However, 5-FC and 5-FU have short half-lives and repeated dosing of 5FC is needed. 5-FC is used as an antifungal agent in humans and is given orally at 1.3 g/m^2 four times daily. It is conceivable that a prolonged, high intratumoral concentration of 5-FU can be maintained by multiple daily administration of 5-FC. Toxicity of TAPET-CD/5-FC, determined by body weight loss, is similar to that of TAPET-CD or 5-FC alone. Combinations of TAPET-CD and 5-FU at effective doses produced higher toxicity, in terms of body weight loss and mortality, than either agent alone. In addition, 5-FU did not increase the anti-tumor efficacy induced by TAPET-CD. 5-FU is not a potent anticancer agent, and continuous infusion is required in human cancer treatment protocols to optimize clinical outcomes. In the present study, frequent dosing with 5-FC was also required to obtain optimal effects. However, continuous infusion of 5-FU in human cancer patients increases toxicity to normal tissues. We believe that TAPET-CD/5-FC provides a solution for generating therapeutic concentrations of 5-FU locally, while avoiding excessive toxicity in normal tissues.

Although the conversion of intratumoral 5-FC to 5-FU is efficient, it likely could be improved. The AUC of 5-FC and 5-FU is 7290 mg · min per kilogram and 2789 mg · min per kilogram, respectively (data not shown), with approximately 30% of 5-FC converted. The TAPET vector uses CD from *E. coli*, which has a higher Km (Kievit *et al.*, 1999) than the fungal CD. It is conceivable that a fungal CD expressed in a TAPET vector could achieve a higher conversion rate and, subsequently, a higher concentration of 5-FU, resulting in a higher antitumor activity. However, increasing CD activity in the TAPET vector could also increase toxicity of the TAPET-CD/5-FC system. A low level of 5-FU was occasionally detected in bone marrow and spleen of mice receiving both TAPET-CD and 5-FC.

The successful use of TAPET-CD/5-FC also suggests the possibility of using attenuated *Salmonella* strains as tumor-specific delivery systems for other protein-based antitumor agents, such as cytokines, antiangiogenic proteins, and prodrug-converting enzymes. *Salmonella* strains have been shown to express a broad spectrum of therapeutic proteins, such as interleukin (IL)-1, IL-2, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α (Saltzman *et al.*, 1996, 1997; Lin, 1999; Yuhua *et al.*, 2001). TAPET-CD is currently being evaluated in cancer patients (Cunningham and Nemunaitis, 2001) as a potential anticancer agent. We are also currently evaluating the expression of various therapeutic proteins in TAPET vectors and the antitumor activities of these therapeutic vectors.

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