The Use of $^{19}$F Spectroscopy and Diffusion-Weighted MRI to Evaluate Differences in Gene-Dependent Enzyme Prodrug Therapies

Daniel A. Hamstra,1,4 Kuei C. Lee,3 Joseph M. Tycheiwicz,3 Victor D. Schepkin,2,4 Bradford A. Moffat,2,4 Mark Chen,1 Kenneth J. Dornfeld,1 Theodore S. Lawrence,1 Thomas L. Chenevert,2,4 Brian D. Ross,2,3,4 Juri T. Gelovani,5 and Alnawaz Rehemtulla1,2,4,*

1Department of Radiation Oncology, 4Department of Radiology, 3Department of Biochemistry, and 2The Center for Molecular Imaging, The University of Michigan Medical Center, Ann Arbor, MI 48109-0582, USA

5Department of Neurology, The Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA

*To whom correspondence and reprint requests should be addressed at The Center for Molecular Imaging, The University of Michigan Medical Center, 1331 East Ann Street, Room 4111, Ann Arbor, MI 48109-0582. Fax: +1 734 763 1581. E-mail: alnawaz@umich.edu.

Available online 19 August 2004

To evaluate noninvasive measures of gene expression and tumor response in a gene-dependent enzyme prodrug therapy (GDEPT), a bifunctional fusion gene between *Saccharomyces cerevisiae* cytosine deaminase (CD) and *Haemophilus influenzae* uracil phosphoribosyltransferase (UPRT) was constructed. CD deaminates 5-fluorocytosine (5FC) to 5-fluorouracil (5FU), and UPRT subsequently converts 5FU to fluorouridine monophosphate, and both of these reactions can be monitored noninvasively in vitro and in vivo using $^{19}$F magnetic resonance spectroscopy (MRS). Following transient transfection the CD–UPRT fusion protein exhibited both UPRT and CD enzymatic activities as documented by $^{19}$F MRS. In addition, an increase in CD activity and thermal stability was witnessed for the fusion protein compared to native CD. Stable expression of CD–UPRT in 9L glioma cells increased both 5FC and 5FU sensitivity in vitro compared to CD-expressing and wild-type 9L cells. Noninvasive $^{19}$F MRS of both CD and UPRT gene function in vivo demonstrated that in animals bearing CD-expressing tumors there was limited conversion of 5FC to 5FU with no measurable accumulation of cytotoxic fluorinated nucleotides (F-nucs). In contrast, CD–UPRT-expressing tumors had increased CD gene activity with a threefold higher intratumoral accumulation of 5FU and significant generation of F-nucs. Finally, CD–UPRT yielded increased efficacy in an orthotopic animal model of high-grade glioma. More importantly, early changes in cellular water mobility, which are felt to reflect cellular death, as measured by diffusion-weighted MRI, were predictive of both durable response and increased animal survival. These results demonstrate the increased efficacy of the CD–UPRT GDEPT compared to CD alone both biochemically and in a preclinical model and validate both $^{19}$F MRS and diffusion-weighted MRI as tools to assess gene function and therapeutic efficacy.

Key Words: cytosine deaminase, uracil phosphoribosyltransferase, 5-fluorouracil, magnetic resonance spectroscopy, diffusion weighted magnetic resonance imaging

INTRODUCTION

Cancer gene therapy using the conversion of pharmaceutologically nontoxic compounds or prodrugs to chemotherapeutic agents has been studied using a number of different gene and drug combinations [1]. One of the most promising gene-dependent enzyme prodrug therapies (GDEPT) utilizes the combination of the enzyme cytosine deaminase (CD) in conjunction with the nontoxic antifungal agent 5-fluorocytosine (5FC) [2,3]. CD deaminates 5FC to the commonly used chemotherapeutic 5-fluorouracil (5FU), which is then converted by a series of intracellular enzymes into 5-fluorouridine-2’-triphosphate (5-FUTP) and 5-fluoro-deoxyuridine monophosphate (FdUMP) [4,5]. 5-FUTP can be incorporated directly into RNA in place of UTP, causing cytotoxicity through the inhibition of both mRNA and ribosomal RNA. In addition, FdUMP is a direct inhibitor of thymidylate synthase, thus secondarily blocking DNA synthesis. An additional advantage of this GDEPT system is that 5FU is readily transported between cells by facilitated diffusion, which allows for a significant “bystander effect” by which one CD-
expressing cell can generate enough 5FU to cause cytotoxicity to multiple surrounding non-CD-expressing cells [6].

The CD/5FC GDEPT was initially evaluated using the Escherichia coli enzyme, and preclinical models varied from growth delay to near-complete responses; however, the durability of response in most cases was limited [6–10]. It was subsequently demonstrated that the biochemical affinity for conversion of SFC to 5FU by CD derived from Saccharomyces cerevisiae is more than 250-fold higher than the identical reaction carried out by the CD derived from E. coli (bCD) [10,11]. The enhanced catalytic activity of yeast CD (yCD) compared to bCD led to increased sensitivity to SFC in vitro in tumors cell lines derived from adenocarcinoma of the colon, squamous cell carcinoma, high-grade sarcoma, or high-grade glioma [10–13]. In addition, this enhanced catalytic activity resulted in both increased tumor growth delay and more sustained response when yCD was compared directly to bCD in preclinical models.

One limitation of both CD-based GDEPT strategies is their reliance upon 5FU, which is rapidly catabolized with nearly 80% of an administered dose of 5FU secreted from the body in an inactive form [4]. Previous efforts to circumvent this metabolism have utilized uracil phosphoribosyltransferase (UPRT) in a GDEPT strategy due to its ability to change 5FU directly to 5-fluorouridine-5-monophosphate (FUMP), thus bypassing the dihydroxyrimidine dehydrogenase-mediated pathway for inactivation of 5FU [14]. Expression of either bacterial or yeast UPRT followed by 5FU treatment resulted in a 15-fold enhancement in cytotoxicity in culture compared to non-UPRT-expressing cells [14,15]. In addition, the combination of both CD and UPRT using a dual GDEPT strategy has been demonstrated either using transduction of both genes on separate adenoviral vectors [16,17] or through a fusion protein between CD and UPRT [15,18]. In both cases there was added efficacy using the dual GDEPT over CD alone. Interestingly, the fusion of the S. cerevisiae CD [22] and the S. cerevisiae UPRT [33] genes gave a dual functional fusion protein that had an unexpected and unexplained increase in CD activity compared to the wild-type CD enzyme [15].

One challenge for the development of gene therapy strategies is the difficulty in monitoring gene expression and function within whole animals. Previously yCD was compared to its bacterial counterpart using 19F magnetic resonance spectroscopy (MRS) by which, both in vitro and in vivo, it was demonstrated to have markedly increased catalytic activity [11,19]. To date similar 19F MRS studies have not been reported characterizing the in vivo activity of UPRT. Diffusion-weighted magnetic resonance imaging (MRI) has also been used to quantify early changes in the diffusibility of tissue water, by which it was demonstrated, both in preclinical models and in patients treated for primary brain tumors, that these changes are correlated with therapeutic efficacy using traditional chemotherapy, the combination of novel biologic therapies and radiation, or GDEPT [13,20,21].

Therefore, in this report we evaluated the interaction between CD and UPRT in GDEPT, by construction of a dual-functional fusion protein between S. cerevisiae CD and Haemophilus influenza UPRT. Like the previously reported CD–UPRT fusion, we find enhanced CD activity both in vitro and in vivo for this fusion. Further, we show that this increased CD activity is secondary to enhanced thermal stability of the fusion protein compared to native CD. Finally, using noninvasive MR techniques, we demonstrate that in vivo expression of CD–UPRT in a high-grade glioma model resulted in increased generation of both 5FU and fluorinated nucleotides (F-nuc) within hours of drug administration and enhanced changes in diffusion-weighted MRI within the first week of treatment, which were both predictive of more sustained tumor growth inhibition. These MRI techniques offer a means to monitor dynamically both gene expression and tumor response in vivo in a fashion that should have direct application for the clinical evaluation of this GDEPT.

RESULTS

Expression of CD–UPRT Results in Both UPRT Activity and Enhanced CD Activity

We constructed the expression plasmid coding for a fusion protein between S. cerevisiae CD and H. influenza UPRT by a direct in-frame fusion of the coding sequence of CD with that of UPRT. We subcloned both CD and the bifunctional fusion gene into the eukaryotic expression plasmid pEF and utilized them for all subsequent experiments (as indicated under Materials and Methods). We transiently transfected these plasmids into COS-1 cells and analyzed them by SDS–PAGE and Western blotting using an anti-yCD antibody. Transfection of the yeast CD expression plasmid (pEF.CD) resulted in the appearance of a band with an apparent molecular weight of 17 kDa (Fig. 1A, lane 2) corresponding to the monomeric form of CD [28], which was not present in cells transfected with a vector control (Fig. 1A, lane 1). In contrast, transfection with the fusion protein expression plasmid (pEF.CD-UPRT) resulted in the appearance of a band with a molecular weight of 46 kDa, corresponding to the expected molecular weight of the monomeric fusion between CD and UPRT (Fig. 1A, lane 3).

To determine if the proteins generated had the expected enzymatic activities, we transiently transfected COS-1 cells with pEF (vector control), pEF.CD, or pEF.CD-UPRT; 48 h later we harvested the cells in assay buffer (as described under Materials and Methods). To account for
required cofactor 5-PRPP was added to the assay buffer the conversion of 5FU to FUMP occurred only when the treated with either 5FU (Fig. 1E) or 5FC (Fig. 1D). Also generate significant quantities of FUMP rapidly when UPRT enzyme, lysates containing CD–UPRT were able to (Fig. 1D). Finally, confirming the presence of an active conversion of 5FC to 5FU following a 45-min incubation exhibited increased CD activity with near-complete and 1C). In contrast, the CD–UPRT fusion protein is consistent with previous reports [23].

Cell lysates generated from vector control-transfected cells had no measurable capacity to convert either 5FC to 5FU or to generate FUMP (data not shown). In contrast, lysates from CD-expressing cells efficiently converted 5FC to 5FU with 50% conversion following a 45-min reaction (Fig. 1B). Like the vector control-transfected cells, CD-expressing samples were unable to generate FUMP when incubated with either 5FC or 5FU (Figs. 1B and 1C). In contrast, the CD–UPRT fusion protein exhibited increased CD activity with near-complete conversion of 5FC to 5FU following a 45-min incubation (Fig. 1D). Finally, confirming the presence of an active UPRT enzyme, lysates containing CD–UPRT were able to generate significant quantities of FUMP rapidly when treated with either 5FU (Fig. 1E) or 5FC (Fig. 1D). Also consistent with the known mechanism of action of UPRT the conversion of 5FU to FUMP occurred only when the required cofactor 5-PRPP was added to the assay buffer (data not shown) [30].

CD–UPRT has Increased Thermal Stability and Deaminase Activity Compared to CD
Previously, a CD–UPRT fusion protein was reported that had markedly enhanced CD activity compared to the native CD [15]. To ascertain if the fusion we constructed exhibited similar behavior we measured the enzymatic activities of CD and CD–UPRT transiently expressed in COS-1 cells (and normalized for expression by Western blot as for Fig. 1) as a function of protein concentration, time, and 5FC concentration. As seen in Fig. 2A CD–UPRT-containing samples were able to convert 5FC to 5FU efficiently and a plateau of conversion was reached using approximately 0.2 μg of normalized cellular lysate (Fig. 2A, closed circles). In contrast, CD-containing cell lysates reached a similar plateau of total conversion but 0.5 μg of extract was required to reach this level (Fig. 2A, open squares). This increase in CD function was dependent upon CD function alone and was not influenced by UPRT activity, for the enhanced conversion of 5FC to 5FU was seen with (data not shown) or without (Fig. 2A) the addition of the required UPRT cofactor PRPP. When we varied the concentration of 5FC to evaluate more carefully the kinetic parameters of the two enzymes over a 2-h reaction at 37°C, it was clear that CD and CD–UPRT had similar apparent K_m values (CD 0.51 ± 0.17 mM and CD–UPRT 0.49 ± 0.10 mM⁻¹), but the apparent V_max of CD–UPRT was increased by twofold compared to CD (CD 1.2 ± 0.2 μmol/min/μg lysate and CD–UPRT 2.5 ± 0.4 μmol/min/μg).

To evaluate further this increased catalytic activity we explored the thermal stability of the two enzymes. Yeast CD has previously been documented to be relatively thermal labile either when purified from baker’s yeast [25] or after overexpression in E. coli [10], and we, therefore, theorized that an increase in enzyme stability might explain the enhanced enzymatic activity seen for CD–UPRT both in this study and in the previous report [15]. We pulse-labeled parallel cultures of transiently transfected COS-1 cells with [35S]methionine/cystine for 5 min and then chased at 37°C with medium containing excess cold methionine/cystine. At various time points we lysed the cells, immunoprecipitated them with anti-yCD antibody, and analyzed them by SDS–PAGE and autoradiography. Quantification of the respective bands using a phosphorimager indicated that CD and CD–UPRT were synthesized at similar initial levels and they exhibited
nearly identical stability of the primary peptide in intact cells, with 73 and 75% of the CD and CD–UPRT peptides still present even after a 4-h chase (data not shown).

To investigate further the thermal stability of both the peptides and their enzymatic activities, we diluted extracts from transiently transfected COS-1 cells containing CD or CD–UPRT in CD assay buffer and then preincubated them at 37°C for increasing periods of time before assaying them by both Western blotting and CD activity assay. CD and CD–UPRT both had similar thermal stability when measured by Western blot with only a slow diminishment in the representative bands, with 85% of CD and 90% of CD–UPRT present even after 24 h incubation (Fig. 2B). Unlike immunoreactivity, however, there were marked differences in enzymatic activity. CD-containing extracts lost >85% of their enzymatic activity after as little as 1 h preincubation at 37°C (Fig. 2C, open squares), with no enzymatic activity appreciated above background after 10 h. Consistent with its increased enzymatic activity, CD–UPRT-containing extracts still maintained >75% of their CD enzymatic activity after a 1-h pretreatment at 37°C (Fig. 2C, closed circles), and even after a 24-h incubation at 37°C they still retained >35% of the starting activity.

CD–UPRT-Expressing Cells are More Sensitive to Both 5FC and 5FU than CD-Expressing Cells

To evaluate the CD and CD–UPRT systems for potential gene therapy of high-grade glioma we stably transfected the 9L rat gliosarcoma cell line with expression plasmids coding for either CD or CD–UPRT. We identified cell lines with similar CD expression by Western blotting and then utilized them for all subsequent experiments (data not shown). Using the sulforhodamine B (SRB) assay for growth inhibition, we found that wild-type and CD-expressing 9L cells had similar sensitivity to 5FU (IC50 2 and 1 μM, respectively; Fig. 3A). Presumably due to the conversion of 5FU to FUMP by UPRT, CD–UPRT-expressing cells were potently sensitized to 5FU, with a 15-fold shift in IC50 to 0.06 μM (Fig. 3A).

Consistent with its role as a nontoxic prodrug, wild-type 9L cells were insensitive to 5FC even at concentrations greater than 3 mM (Fig. 3B). CD-expressing 9L cells were >1000-fold more sensitive to 5FC, with

FIG. 2. CD and CD–UPRT were transiently transfected into COS-1 cells and extracts normalized for CD expression were then analyzed for their capacity to convert [3H]5FC to [3H]5FU. (A) Percentage of conversion of 5FC to 5FU following 2 h reaction at 37°C using increasing quantities of extracts containing CD (open squares) or CD–UPRT (closed circles). (B) Western blots of CD- and CD–UPRT-containing cell lysates preincubated at 37°C for 0, 1, 2, 4, 10, or 24 h. (C) Percentage of remaining deaminase activity normalized to starting activity following preincubation at 37°C for increasing periods of time for CD (open squares) or CD–UPRT (closed circles). Data represent the means and standard errors of at least four experiments.

FIG. 3. SRB growth-inhibition assays for treatment of wild-type (squares), CD-expressing (circles), or CD–UPRT-expressing (triangles) 9L cells treated with (A) 5FU or (B) 5FC. Data represent the means and standard errors of at least eight replicate wells.
an IC\textsubscript{50} of 3 \textmu M, which approached their sensitivity to 5FU (Fig. 3B). Finally, analogous to their increased sensitivity to 5FU, CD–UPRT-expressing 9L cells were >15,000-fold more sensitive to 5FC than wild-type 9L cells and 15-fold more sensitive to 5FC than even CD-expressing cells (IC\textsubscript{50} 0.2 \textmu M, Fig. 3B). In fact, these cells were sensitive to 5FC at concentrations even lower than that needed for growth inhibition of wild-type cells with 5FU.

Expression of CD–UPRT Leads to Enhanced Accumulation of 5FU and F-Nucs in Vivo

To confirm that the enhanced enzymatic activity and growth inhibition identified in culture were also true in vivo and to establish the ability to monitor noninvasively both CD and CD–UPRT gene function, we implanted 9L cells stably expressing CD or CD–UPRT subcutaneously in the hindlimb of nude mice. Once tumors had developed to a mean size of 100–150 mm\textsuperscript{3} we treated the mice with a single intraperitoneal injection of 5FC (1000 mg/kg) and used \textsuperscript{19}F MRS to follow the metabolism of 5FC, 5FU, and F-nucs. Given the location of the tumor within the hindlimb and the extended position of the leg with an intervening plastic restraint device there was no measurable signal acquired at the surface coil from the 5FC within the abdominal cavity. Fig. 4A represents stacked \textsuperscript{19}F MR spectra in non-tumor-bearing muscle from a representative single animal for which each individual spectrum represents a sequential 20-min acquisition over the 5-h time course. In non-tumor-bearing muscle, 5FC accumulated to a peak 60 min following ip injection and then slowly dissipated over the 5 h of the experiment without any conversion to either 5FU or the fluorinated metabolites (Fig. 4A). Rapid conversion of 5FC to 5FU was identified in CD-expressing 9L tumors in which even at the second time point (40 min) following ip injection there were measurable amounts of 5FU (Fig. 4B). This 5FU continued to accumulate over the 5 h of the experiment, but never to more than 30\% of the peak 5FC concentration. In addition, there were no MRS measurable amounts of F-nucs. Finally, consistent with the increased CD activity documented in vitro, in a CD–UPRT-expressing tumor (Fig. 4C) there was much more efficient conversion of 5FC to 5FU such that later in the experiment all of the 5FC was consumed. In addition, there were measurable levels of the F-nucs present as early as 100 min after injection of 5FC, and these continued to accumulate throughout the experiment. Previous reports with \textsuperscript{19}F MRS following 5FU treatment have documented that the F-nucs peak identified by MRS is made up of approximately 50\%FdUMP and, therefore, represents intracellular trapping of the active metabolites, which is predictive of response to 5FU treatment [23]. Despite the high levels of 5FU and F-nucs that were generated within the CD–UPRT-expressing tumor there was no accumulation of 5FU in the contralateral hindlimb (Fig. 4A), and none of the mice demonstrated weight loss or other overt signs of 5FU toxicity.

The spectra in Fig. 4 represent the dynamic pharmacokinetics of 5FC, 5FU, and F-nucs in individual animals. In comparison, Figs. 5A and 5B show the population kinetics for all of the animals in the experiment, in which a peak 5FC concentration of slightly greater than 2.5 mM was seen in both CD- (n = 3) and CD–UPRT- (n = 9) expressing tumors 100 min following ip injection. In non-CD-expressing muscle 5FC reached a similar peak at the 100-min time point and was eliminated with apparent first-order kinetics with a half-life of approximately 180 min (data not shown). In CD-expressing tumors we observed a similar half-life of 185 min, which likely reflects predominantly diffusion of native 5FC out of the tumor with limited deamination to 5FU. In CD–UPRT-expressing tumors there was a significantly higher conversion of 5FC to 5FU, which was reflected in a shortening of the intratumoral half-life of 5FC to 120 min.
Importantly, the peak 5FC concentrations observed are all well above the $K_m$ (~0.5 mM) observed for both enzymes in vitro (data presented above and in Refs. [10] and [11]).

In CD-expressing tumors on average 5FU accumulated to a plateau (0.5 mM), which was one-fifth of the peak 5FC concentration (2.5 mM), after approximately 60 min and remained at this level throughout the 5-h course of the experiment (Fig. 5A). Similar to the increased deaminase activity identified in vitro, in CD–UPRT-expressing tumors there was a significant increase in the concentration of 5FU, which reached a level threefold higher than that seen in CD-expressing tumors (1.5 mM vs 0.5 mM), with a plateau reached after slightly greater than 2 h (Fig. 5B). This represents peak intratumoral 5FU concentrations equal to approximately 55% of the peak SFC concentration. More importantly, the concentrations of F-nucs, which likely represent the cytotoxic metabolites, first became detectable above background 80 min after injection of SFC and continued to accumulate to a plateau concentration of 0.5 mM after approximately 3 h. The F-nucs then remained stable for the remainder of the experiment.

CD–UPRT Leads to Increased Changes in Diffusion-Weighted MRI and Prolonged Animal Survival

Having determined that CD–UPRT produced both greater sensitivity to SFC in vitro and more efficient conversion to 5FU in vivo we next evaluated its efficacy in an orthotopic animal model of high-grade glioma. We implanted wild-type or CD- or CD–UPRT-expressing cells stereotactically into the right frontal lobe of Fischer rats. We verified the presence of tumors using T2-weighted MRI with tumors first visible approximately 10 days following injection. Prior to the initiation of treatment the three different cell lines produced tumors with similar doubling times of approximately 70 h. When tumors reached a mean volume of 80–100 $\mu l$ (day 17 postimplantation) we treated control animals with daily ip injections of saline and animals bearing CD- or CD–UPRT-expressing tumors with daily ip injections of SFC (1000 mg/kg) for 10 days.

Figs. 6A through 6C represent sequential images acquired over the course of treatment for individual representative animals bearing control, CD-expressing, or CD–UPRT-expressing tumors. The anatomical data from the T2-weighted scans are layered with a color wash representing the apparent diffusion coefficient (ADC) values. Initial tumors are of similar size and have ADC values that are similar, although not identical, representing the heterogeneity evident between different tumors. Plots of mean tumor volume (Fig. 7A) and percentage change in mean ADC for all of the animals in the groups (Fig. 7B) are also depicted. Thus, the ADC maps in Figs. 6A–6C allow one to visualize individual anatomic differences in ADC both at baseline and in response to treatment not only between animals, but also within different areas within an individual tumor. In contrast, the curves in Fig. 7B represent the percentage change from baseline for the mean ADC for all animals in the three different treatment groups. This, therefore, facilitates comparisons between treatment groups although precise anatomic detail in lost in this manner.

Animals bearing control tumors exhibited rapid tumor growth (Figs. 6A and 7A) with a tumor doubling time of approximately 72 h. In addition, consistent with a lack of therapeutic intervention, there was no change in the ADC, with large viable tumors growing throughout the course of the experiment. In animals bearing CD- or CD–UPRT-expressing tumors daily treatment with SFC resulted in a significant delay in tumor growth, with tumors completing at most one additional doubling
before beginning to regress (Figs. 6B, 6C, and 7A). The difference in tumor growth rate between the two treated groups and the control animals was evident as early as 4.5 days after the initiation of treatment ($P < 0.042$). However, despite the increased sensitivity to SFC witnessed for CD–UPRT-expressing cells in culture and the enhanced production of F-nucS demonstrated in vivo, there were no statistically significant differences in tumor growth rate between CD- and CD–UPRT-expressing tumors throughout the course of treatment. In both groups tumors regressed until they were undetectable by T2 MRI approximately 21–24 days following the start of treatment. Despite these similar rates of regression during treatment and apparent radiographic complete responses in all treated animals, two of three animals bearing CD-expressing 9L tumors had recurrence of multiple foci in and around the initial tumor volume beginning 3–4 weeks after the start of treatment. These recurrences grew rapidly with a tumor doubling time consistent with the pretreatment rates and displayed an ADC value consistent with viable and growing tumor (data not shown). In contrast, all six animals in the CD–UPRT group had complete radiographic regression of tumor by 21 days after the start of treatment, and no measurable recurrence of tumor could be documented in the animals bearing CD–UPRT-expressing 9L tumors even out to 60 days after the start of treatment.

Unlike volumetric measurements, which were not different between the treated groups and also were not predictive for the durability of response, diffusion-weighted MRI revealed early differences not only between the treated groups and the control animals, but also between the two treatment arms (Figs. 6A–6C and 7B). As early as 4.5 days after treatment was started, both the CD- and the CD–UPRT-expressing tumors exhibited statistically significant differences in ADC compared to those in control animals (Fig. 7B, $P < 0.05$); further, at this time the increase in diffusion

**FIG. 6.** Individual T2-weighted coronal MRI through the largest cross section of tumor acquired at the first day of treatment (day 1) and serially afterward. The map of apparent diffusion coefficients (ADC units of $10^{-1}$ mm$^2$/s) is overlaid in a color wash, with blue representing low diffusion and red high diffusion. (A) Wild-type 9L tumor treated with PBS. (B) CD-expressing or (C) CD–UPRT-expressing tumors treated with daily SFC injections (1000 mg/kg for 10 days).
exhibited by CD–UPRT-expressing tumors was not only different from that of control animals but also different from that of CD-expressing tumors. At this time point CD-expressing tumors had a 12.2% increase in relative ADC compared to a 24.8% increase in the relative ADC for CD–UPRT-expressing tumors (P = 0.045). Throughout the course of treatment, the CD–UPRT-expressing tumors exhibited a more robust and rapid rise in ADC, reaching a plateau with a 110% increase in ADC at day 10 of treatment. CD-expressing tumors, in contrast, had a less rapid and dramatic rise in ADC, reaching a plateau at day 10, which was less than 35% increase from the starting value.

Similar to previous studies [13,26,32], control animals bearing 9L tumors (n = 6) had a median survival of 25 days after implantation, with all animals succumbing or being euthanized secondary to cerebral tumors by day 27 (Fig. 8). Animals bearing CD-expressing tumors exhibited a small but significant 10-day increase in median survival to 35 days, with one of three animals alive and disease free at day 80 postimplantation (P < 0.01 compared to control animals). As predicted by both the rapidity and the extent of response (by relative ADC change) and the durability of response (by conventional MRI) CD–UPRT afforded a significant enhancement in overall and cause-specific survival, with all treated animals (n = 6) alive and free of disease for up to 80 days (P = 0.007 compared to control). Further, there was no evidence of recurrent or residual disease in any of the animals at this time by MRI or postmortem necroscopy. This treatment was also significantly better than that seen for animals treated with CD alone (P = 0.03).

**DISCUSSION**

High-grade primary brain tumors represent a challenging clinical disease, for despite advances in surgical technique, radiation therapy, and new chemotherapeutic agents the median survival after diagnosis is still less than 12 months [27]. The vast majority of patients die of local disease either through lack of response to therapy or through recurrence within the initial primary site. Despite the locally aggressive nature, metastatic disease, either within the brain or systemically, is extraordinarily rare. For this reason techniques to increase the intensity of local treatment have been tried, including aggressive surgical resection, high-dose radiation therapy, intensified systemic or local–regional chemotherapy, and multimodality treatments, which often utilize a combination of several of these therapies [27]. Due to their ability to generate high local concentrations of a toxic compound from a pharmacologically inert precursor, GDEPT strategies can be viewed as one further step along the progression of these aggressive local therapies [28].

[FIG. 7. (A) Tumor volume and (B) percentage change in mean ADC values from baseline as a function of time posttreatment for wild-type 9L tumors treated with PBS (closed triangles) or for CD-expressing (closed circles) or CD–UPRT-expressing (open circles) 9L tumors treated with SFC (1000 mg/kg ip daily for 10 days starting at day 1). Data represent the means and standard errors for wild-type (n = 6), CD-expressing (n = 3), and CD–UPRT-expressing (n = 6) tumors.

[FIG. 8. Kaplan–Meier survival curve for animals as a function of time since implantation for wild-type 9L tumors treated with PBS (dashed line, n = 6) or for CD-expressing (dashed-dotted line, n = 3) or CD–UPRT-expressing (solid line, n = 6) 9L tumors treated with SFC (1000 mg/kg ip daily for 10 days starting at day 17 postimplantation).]
The combination of CD and SFC in a GDEPT strategy is one of the more promising cancer gene therapy strategies being evaluated. It takes advantage of an enzymatic activity not found in mammalian cells [2], a relatively inert prodrug that has both oral bioavailability and excellent penetrance into the cerebral spinal fluid [5], and, finally, a target cytotoxic compound that has both broad oncologic applicability and well-studied clinical and pharmacologic characteristics [4]. Treatment with high-dose 5FU is limited by both hematogenous and mucosal toxicity, and, therefore, the direct production of 5FU at the tumor site may be able to generate dose intensification without the associated increase in systemic toxicity. Early work with bCD in combination with 5FC was promising; however, later studies revealed that due to poor catalytic efficiency this system likely has limited clinical applicability [8–11]. However, the more catalytically active cytosine deaminase derived from S. cerevisiae has been documented to enhance both SFC cytotoxicity and tumor growth inhibition in multiple tumor models both with and without concurrent radiation therapy [11–13,29].

Given that SFU is itself a prodrug, which must undergo metabolic activation to generate the cytotoxic fluorinated nucleotides (5-FUTP and FdUMP), it is not surprising that UPRT showed sensitization when combined with SFC treatment using GDEPT [14]. Further, in previous reports through the combination of both CD and UPRT an added level of enzymatic activation was achieved, which further sensitized transduced cells to SFC [15–17]. Interestingly, when a direct fusion protein between S. cerevisiae CD [22] and S. cerevisiae UPRT [33] was evaluated there was an unexpected increase in the deaminase activity of the fusion protein, which was 5- to 100-fold higher than the native yCD [15]. Following adeno viral transduction of human tumor cell lines in culture this CD–UPRT fusion resulted in significantly higher conversion of SFC to SFU than either yCD alone or yCD infected in trans with UPRT. Similarly, we here demonstrate an increase in the catalytic activity of yCD when expressed as a fusion protein with H. influenza UPRT. To elucidate the difference in this activity we explored the thermal stability of CD–UPRT, since yCD had previously been reported to be thermal labile relative to bCD [10,25]. Interestingly, under the conditions tested here, within 2 h yCD had lost >80% of its catalytic activity without any significant change in protein level, while CD–UPRT retained virtually all of its enzymatic function at this time. Kinetic analysis revealed that the two enzymes have similar \( K_m \) values for SFC, which are consistent with the previously reported values for yCD [10] and within the achievable serum concentrations [30]. However, CD–UPRT had a slightly greater than 2-fold increase in \( V_{max} \) relative to yCD, which may simply be a reflection of the added thermal stability of CD–UPRT over the 2 h of the assay, since the magnitude of this difference was less when shorter reactions were analyzed (data not shown).

Interestingly, the added thermal stability of this fusion protein is not a universal phenomenon, but instead it may be related to increased dimerization of CD–UPRT by the well-documented formation of UPRT multimers [24,31]. We have not seen similar increases in thermal stability in other yCD fusion proteins that are not expected to cause multimerization (D.A.H. and A.R., unpublished data). In support of this theory, it is worth noting that the UPRT from E. coli (which bears 90% amino acid identity to H. influenza UPRT) is markedly thermal stable, retaining almost 100% of its catalytic activity even after heating to 65°C [32]. There is less homology between the S. cerevisiae and the H. influenza UPRT enzymes (<40%), so it is much more difficult to extrapolate whether the increased catalytic efficiencies seen in our fusion and the one reported by Erbs et al. [15] are both because of similar stabilization (although preliminary data that we (D.A.H. and A.R.) have generated would support a similar increase in thermal stabilization for the yeast–yeast fusion). It is also curious to note that the added deaminase activity documented for the two CD–UPRT fusions incorporating the yeast enzyme was not observed when the intrinsically more thermal stable E. coli CD was fused to E. coli UPRT [18]. This, therefore, may lend further credence to the theory that this fusion is not more enzymatically active per se; rather, it simply has enhanced thermal stability allowing it to function for a greater period of time.

Another possible mechanism for the increased deaminase catalytic efficiency of CD–UPRT is through the release of product inhibition, for guanyl and thymidyl nucleobases are both potent mixed competitive/non-competitive allosteric inhibitors of S. cerevisiae CD, raising the possibility that SFU may act in a similar role [30], and by rapidly converting 5FU to FUMP this inhibition is released in CD–UPRT. However, the fact that the added CD activity was documented in vitro both with and without the required UPRT cofactor PRPP suggests that this is not the case. Nevertheless, the added deaminase function of CD–UPRT is not simply confined to in vitro studies, for using \( ^{19}F \) MRS we observed increased deamination of SFC in vivo. Similar to the increased CD activity in vitro, tumors expressing CD–UPRT were able to generate threefold higher concentrations of 5FU than CD-expressing tumors despite similar levels of CD enzyme.

The likely limiting step in any GDEPT strategy is the presence of a potent bystander effect, by which one transduced cell can kill many nontransduced neighboring cells. Currently available vectors for in situ gene transduction are limited in the total percentage of cells that can be transduced (often 10% or less of a tumor mass) [28]; thus without a potent bystander effect there...
will be limited anti-tumor efficacy. One might be concerned that de novo expression of UPRT would lead to trapping of FUMP, which unlike 5FU is not freely membrane permeable, and thus this fusion protein would actually lead to decreased cytotoxicity in nontransduced neighboring cells [34]. The “factory” might be killed before it has sufficient time to produce enough 5FU to kill neighboring cells, thereby decreasing the bystander effect. However, the added CD activity documented both in vitro and in vivo revealed that even after 30% conversion of 5FU to FUMP there was still a two- to threefold higher concentration of 5FU in CD–UPRT-expressing tumors than in CD-expressing tumors. Thus, a net increase in 5FU within the tumor is observed even after taking into account trapping of SUMP. This “dose intensification” achieved through increased concentrations of 5FU generated for CD–UPRT-expressing cells compared to CD-expressing cells would likely provide a therapeutic benefit on its own, but the addition of UPRT added another amplification in the cytotoxicity through an increase in the generation of the fluorinated nucleotide metabolites of 5FU, which are the true cytotoxic moieties [4].

Evidence for the importance of the generated F-nucs is seen in a previous report using MRS in animal models, which looked at the modulation of 5FU metabolism through the coadministration of allopurinol, which inhibits FUMP formation indirectly by orotate phosphoribosyl transferase through depletion of the required cofactor PRPP [35]. In tumors depleted of PRPP by pretreatment with allopurinol subsequent administration of 5FU resulted in similar 5FU peaks by MRS but complete loss of the F-nuc peak and abrogation of both tumor and systemic cytotoxicity. Indeed, in that report, the level of F-nucs generated within the tumor and not 5FU dose was correlated directly with response. Similarly, our previous work with bCD- and yCD-expressing HT29 colon carcinoma tumors in vivo also demonstrated differences in the accumulation of intracellular 5FU and F-nucs that correlated with response [19]. In that study bCD-expressing tumors generated little 5FU and only yCD-expressing tumors were able to generate measurable quantities of both 5FU and F-nucs along with significant tumor responses. The differences in F-nucs accumulation in yCD-expressing cells between that report and our current study likely reflect basal differences in uridine phosphorylase expression, which is known to be more highly expressed in intestinal tissues [36].

Thus, the results presented here for the first time reveal that both CD and UPRT functions can be noninvasively monitored in vivo using 19F MRS. Further, the intracellular pharmacokinetics of 5FC, 5FU, and F-nucs may be able to predict response to this GDEPT strategy as early as several hours after treatment with 5FC. More excitingly early work with 19F magnetic resonance imaging of 5FU and its metabolites may also eventually be able to give both spatial and temporal information about gene function to guide subsequent therapy [37].

One advantage of using MRI to follow tumor growth is that treatment was performed on established tumors more than 2 weeks after implantation of cells to simulate the treatment of unresectable high-grade glioma. This is likely more reflective of clinical treatment than starting 5FC treatment only 5 days after tumor cell implantation, when established tumors have not developed [17]. By T2-weighted MRI there was a significant slowing of growth for both CD- and CD–UPRT-expressing tumors within the first several days after starting 5FC treatment compared to control animals, and all treated animals showed complete radiographic response 2 weeks after the completion of a 10-day course of 5FC. However, despite this fact only the animals bearing CD–UPRT-expressing tumors had a durable response, with no evidence of tumor recurrence even more than 2 months after the completion of therapy. Thus, traditional volumetric measures of tumor growth and regression were unable to predict this significantly different outcome.

Like MRS measures of fluorine metabolism, diffusion-weighted MRI may be another early means to predict noninvasively tumor response to both traditional oncologic therapies and to gene therapy [13,20]. Volumetric measures by MRI are limited because they do not carry any significant functional information about the tissue outlined within the volume of interest. Healthy viable tissue is scored as the same volume as dying or even necrotic cells. Thus, it is not surprising that simple volumetric measures were unable to differentiate between the significantly different outcomes observed for the CD- and the CD–UPRT-expressing tumors following 5FC treatment. Diffusion-weighted MRI is a relatively new technique that measures the free mobility of water within tissues [20,38,39]. In normal healthy tissues a large proportion of water is confined within the intracellular environment and has a restricted diffusion coefficient (i.e., a low ADC value). However, when cells begin to die by either apoptosis or necrosis there is a disruption of cellular membranes and an alteration of this intracellular water pool with a corresponding increase in water diffusion (i.e., a high ADC value). Since changes in water diffusion precede volumetric responses this alteration in the apparent diffusion coefficient can thus often predict a tumor response prior to changes in volume, and differences in ADC for similar volume tumors may also predict differences in the viability of the tumor cells.

Despite a lack of difference in tumor regression (as determined by T2-weighted MRI) during treatment between CD- and CD–UPRT-expressing tumors, there was an early and dramatic difference in diffusion-
weighted MRI between these two arms. As early as 4.5 days after starting therapy there were statistically significant differences in relative ADC between CD- and CD-UPRT-expressing tumors, and this difference in ADC continued to increase throughout the course of therapy and was maintained until no viable tumor was visible by T2-weighted images or diffusion scans. Thus, diffusion-weighted imaging (DWI) predicted an increased cytotoxic effect in the CD–UPRT-expressing tumors that could not be predicted based on simple volumetric scans; further, this was borne out with a 100% long-term survival in animals bearing CD–UPRT-expressing tumors, while two-thirds of the CD-expressing animals eventually succumbed to recurrent disease. Although there were a limited number of animals in the CD treatment arm in this study the results obtained in terms of volumetric changes, DWI elevation, and long-term survival are similar to those we previously reported using the same model system looking at CD gene therapy alone, lending greater support to the comparison with CD–UPRT [13].

This increased sensitivity of diffusion-weighted MRI versus conventional imaging has been offered in support of this technique as an early and more sensitive assay for response to a number of antineoplastic therapies [38,39]. We previously demonstrated not only that changes in ADC values between individual animals could predict both response to therapy and prolonged survival, but also that differences in ADC response within individual tumors were predictive of local heterogeneities within the tumor [20,26]. We have also previously demonstrated that ADC is correlated directly with cellular density as determined by histological analysis and is thus a true predictor of response to therapy [13,20,26]. These observations have further been borne out preliminarily in patients treated with conventional chemotherapy or radiation therapy for primary brain tumors in which responses both between patients and within individual tumors could be correlated with ADC changes during treatment. Thus changes or lack thereof in ADC may help guide subsequent treatment decisions with areas not showing a response given further treatments with a GDEPT or boosted with an additional therapy, such as external beam radiation therapy. In contrast, areas with marked changes in ADC predictive of good response may be spared from further therapy, thus decreasing the potential toxicities of additional therapy.

In conclusion, this study demonstrates that enhanced thermal stability is the underlying mechanism for the increased CD catalytic efficiency of CD–UPRT compared to CD. In addition, using $^{19}$F MRS we, for the first time, document gene function in vivo for both CD and UPRT quantitatively in real time and also demonstrate that the enhanced CD activity demonstrated for CD–UPRT in vitro is maintained in vivo. Further, the increased enzymatic function for CD–UPRT translates into increased efficacy as documented by diffusion-weighted MRI and sustained tumor regression. More importantly, these MR techniques are not limited solely to this model system, but likely can be expanded to clinical use, and we hope that through improved understanding of both gene expression and function, along with measures of early response to therapy, they may promote our understanding and use of GDEPT or other gene therapies for the treatment of malignancy.

**MATERIALS AND METHODS**

**Expression plasmids.** The expression cassette for *S. cerevisiae* CD as previously described [11] was subcloned into the mammalian expression vector pCMV (Roche Diagnostics Institute, Cambridge, MA, USA) and drives gene transcription via the EF1-a promoter and contains the neomycin-resistance gene downstream of an internal ribosomal entry site. The *H. influenza* UPRT gene was amplified from bacterial genomic DNA by PCR (Expand Hi-Fidelity DNA Polymerase, Boehringer GmbH, Mannheim, Germany) using the published sequence [40]. The CD and UPRT genes were then fused in-frame following deletion of the stop codon in CD using overlap PCR [41]. The final plasmids were confirmed by direct sequencing at The University of Michigan DNA Sequencing Core.

**Cell culture and protein expression.** Rat 9L gliosarcoma cells, obtained from the Brain Tumor Research Center (University of California, San Francisco, CA, USA), and COS-1 African green monkey kidney cells (obtained from the Genetics Institute) were grown as monolayers in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO$_2$. Cells were transfected with the appropriate expression plasmids using FuGene6 (Roche Diagnostics Corp., Indianapolis, IN, USA) per the manufacturer’s instructions. For transient transfection cells were utilized for the appropriate assays 48–72 h after transfection. For generation of stable cell lines 48 h after transfection cells were passed at a density of 1 cell/well in 96-well dishes in the presence of G418 (200 µg/ml; Invitrogen, Carlsbad, CA, USA) for 2 weeks to select stably expressing clones. Individual clones were characterized by Western blotting and activity assays as indicated below.

**Protein analysis.** For Western blots cells were harvested in NP-40 lysis buffer (25 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40) supplemented with Complete Protease Inhibitors (Roche Diagnostics Corp.). Lysates were normalized for protein content using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) and then analyzed by SDS-PAGE and Western blotting using protocols previously described using a rabbit anti-CD antibody (1:1 × 10$^6$ dilution [10]) followed by goat anti-rabbit antibody conjugated to horseradish peroxidase (Fischer, Pittsburgh, PA, USA) and visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

**CD-activity assays.** Cells expressing CD or CD–UPRT were harvested in assay buffer (100 mM Tris, pH 7.8, 150 mM NaCl, 1 mM EDTA) and freeze–thawed three times. Prior to activity assays the level of CD or CD–UPRT expression was normalized by serial dilution and Western blotting followed by quantification using NIH Image software. CD activity was measured as percentage conversion of $^{3}$H-labeled 5FC to 5FU using the protocol described previously [11,42]. Briefly, 100 mM SFC was spiked with 0.5 mM S-[6-3H]JFIC (1 μCi/mmol; Moravek Biochemicals, Brea, CA, USA) and then diluted with buffer, yielding final concentrations of cold SFC from 0.05 to 20 mM. Each assay was performed in a 30-µl reaction volume containing 0.02 to 10 µg of protein extract. Assays were incubated at 37°C for 2 h at which time they were quenched by the addition of 1 M...
acetic acid. The 5-\(^{14}\)HFU produced was isolated by elution from an SCX Bond Elute Column (Varian, Harbor City, CA, USA) and counted on a liquid scintillation counter. Data were plotted using GraphPad Prism software (GraphPad Software, San Jose, CA, USA) and apparent \( K_m \) and apparent \( V_{\text{max}} \) values were calculated based on nonlinear regression analysis. All assays were performed three or four times on samples derived from at least two separate transfections.

**Growth Inhibition assay.** The SBB assay was utilized to measure growth inhibition of the respective 9L cell lines in the presence of SFC or SFU. Briefly, \( 18 \) h prior to treatment cells were plated at a density of 1000 cells/well in 96-well tissue culture plates. Medium was then changed to that containing SFC or SFU (both obtained from Sigma) and cells were treated for 72 h at which point cells were fixed and stained according to Skehan et al. [43]. Plates were analyzed on a microtiter plate reader using a 490-nm filter (\( V_{\text{max}} \) Molecular Devices, Sunnyvale, CA, USA). Data plotted represented the mean and standard errors of at least eight replicate wells.

**Animal experiments.** All experiments were approved by the University of Michigan Committee for Animal Use. For in vivo spectroscopy CD- or CD-UPRT-expressing 9L cells growing in log phase were trypsinized and counted by Coulter counter and trypan blue at which time 1 \( \times 10^9 \) viable cells were implanted subcutaneously into the hindlimbs of CD-1 nu/nu mice (The Jackson Laboratory, Bar Harbor, ME, USA). When tumors had grown to a mean size of 100–150 mm\(^3\) animals were treated with a single ip dose of SFC (1000 mg/kg, 15 mg/ml) prior to being followed by spectroscopy.

Intracerebral 9L tumors were induced in male Fischer 344 rats weighing between 125 and 150 g as previously described [44]. Briefly, 9L cells (1 \( \times 10^5 \)) were implanted in the right forebrain at a depth of 3 mm through a 1-mm Burr hole. The surgical field was cleaned with 70\% ethanol, and the burr hole was filled with bone wax to prevent the extracerebral extension of the tumor. SFC was given as a solution in normal saline by ip injection (1000 mg/kg for 10 days) except for control 9L tumor animals, which received saline-only injections.

**Magnetic resonance spectroscopy.** In vitro and in vivo MRS were performed essentially a previously described using a Varian Unity Inova system equipped with a 9.4-T scanner tuned to 376 MHz [19]. For in vitro analysis cell lysates containing CD or CD-UPRT were resuspended in assay buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 5 mM MgCl\(_2\), and 10 mM 5-Phospho D (PPi), Sigma), which was supplemented with 2.5 mM SFC or SFU. Samples were incubated at 37°C for increasing times at which aliquots were removed, heat-inactivated at 95°C for 10 min, and then subjected to MRS.

For in vivo spectroscopy, animals were analyzed as previously described. Briefly, the mice were anesthetized with isoflurane and then injected ip with SFC (1000 mg/kg) prior to being positioned in a specially designed plastic jug that immobilizes the tumor-bearing limb in an extended fashion away from the body. The sedated mouse was then placed so that the tumor was directly under the surface coil and at the isocenter of the magnet. Spectra were then acquired and averaged over 20 min for a minimum of 3 h using a single pulse with a TR of 4 s and a signal width of 10 MHz. To obtain absolute intratumoral metabolite concentrations, a scaling factor relative to a Na\(^+\) internal control was used. The scaling factor was obtained from MRS experiments by using the NaF standard microcell placed adjacent to the coil and a tumor phantom containing a known concentration of SFC in normal saline.

**Magnetic resonance imaging.** On day 12 post-intracerebral implantation of 9L cells initial diffusion and T2-weighted images were acquired on all animals as previously described [13, 28]. Animals were imaged every 2–3 days throughout the course of the experiment. Rats were anesthetized with an isoflurane/air mixture and maintained at 37°C inside the magnet using a heated, thermostatted circulating water bath. A single-slice gradient-echo sequence was used to confirm proper animal positioning and to prescribe subsequent imaging. An isotropic, diffusion-weighted sequence was employed with two interleaved b factors (\( 3b = 1148 s/mm^2 \)) and the following acquisition parameters: TR/TE 3500/60 ms, 128 \( \times \) 128 matrix, and 3-cm FOV. Thirteen 1-mm-thick slices separated by a 0.2-mm gap were used to cover the whole rat brain. The gradient first moment was zeroed to reduce the dominant source of motion artifact; a 32-point navigator echo was added to each phase-encoded echo. The phase deviation of each navigator echo relative to its mean was subtracted from the respective images prior to the phase-encode Fourier transform. The low b-factor images were essentially T2-weighted to allow tumor volume measurements, as previously described. Isotropic ADC maps were calculated for each image set and ADC pixel values were converted to color overlays onto the T2-weighted images.

Tumor volumes were assessed on late-echo images in which the tumors appear hyperintense. These images were acquired as part of the diffusion-weighted scan (low b-factor images, TR/TE = 3500/60 ms) or in separate standard T2-weighted series (TR/TE = 3000/80 ms). The tumor boundary was manually defined on each slice using a region-of-interest tool and then integrated across slices to provide volume estimates. Tumor doubling time was determined by a linear least-squares fit to the logarithm of tumor volume using all posttreatment (or sham treatment) time points.

**Data analysis.** Statistical comparisons were performed using two-tailed Student’s t-test function within Microsoft Excel except for Kaplan–Meier probability for survival analysis, which was done using GraphPad Prism (GraphPad Software).

**Acknowledgments**

Support from this work was provided by NIH Grants P01CA85878, P05CA01014, RO1CA83104, and R24CA83099; The University of Michigan SPORE in Head and Neck Cancer; and a Varian Medical Systems/RSA Holman Pathway Resident Research Award.

**REFERENCES**


