Abstract

The successful translation of gene therapy for clinical application will require the assessment of transgene activity as a measure of the biological function of a therapeutic transgene. Although current imaging permits the noninvasive detection of transgene expression, the critical need for quantitative imaging of the action of the expressed transgene has not been met. In vivo magnetic resonance spectroscopic imaging (MRSI) was applied to quantitatively delineate both the concentration and activity of a cytosine deaminase–uracil phosphoribosyltransferase (CD-UPRT) fusion enzyme expressed from a transgene. MRSI enabled the generation of anatomically accurate maps of the intratumoral heterogeneity in fusion enzyme activity. We observed an excellent association between the CD-UPRT concentration and activity and the percentage of CD-UPRT+ cells. Moreover, the regional levels of UPRT activity, as measured by imaging, correlated well with the biological effect of the enzyme. This study presents a translational imaging paradigm for precise, in vivo measurements of transgene activity with potential applications in both preclinical and clinical settings. [Cancer Res 2008;68(8):2878–84]

Introduction

The pharmacokinetic and pharmacodynamic properties of a drug determine its therapeutic efficacy in vivo (1). This association holds true for all conventional drugs and suggests that the activity of novel therapeutic strategies, including gene therapy, must be thoroughly characterized to achieve therapeutic effect (2). In the excitement to introduce gene therapy for clinical trial, the quantitative characterization of transgene activity has been underemphasized (3, 4). The inability to translate the successes of preclinical gene therapy studies into clinical treatments has underscored this deficiency in quantitative data (5). We show quantitative in vivo magnetic resonance (MR) spectroscopic imaging (MRSI) of the regional activity and concentration of an exogenous enzyme expressed from a therapeutic transgene. We further establish that MRSI measurements of regional transgene activity can provide an effective index of the biological function of this enzyme. The ability of MRSI to facilitate the in vivo assessment of transgene activity suggests a potential role for this technique in optimizing the implementation of gene therapy.

To date, noninvasive imaging approaches used to monitor gene therapy strategies have primarily focused on semiquantitative assessments of transgene expression (6–11). In contrast, the delineation of the regional activity of a transgene is expected to enable the functional evaluation of gene therapy. Transgene activity can be assessed through serial, quantitative, and noninvasive measurements of both the substrate specific for the transgene and its resultant metabolite(s). For example, the cytosine deaminase–uracil phosphoribosyltransferase (CD-UPRT) fusion enzyme metabolizes 5-fluorouracil (5-FU) and 5-fluorocytosine (5-FC) to discernible anabolites (refs. 12, 13; Fig. 1). MRSI is well suited for this application as it allows the absolute quantitation of transgene specific substrates and metabolites with spatial encoding that provides the opportunity to detect local variations in the target tissue (14, 15). These substrates and metabolites may be distinguished in the acquired images based on their relative chemical shifts. In enabling the absolute quantitation of these resonances, MRSI provides the unique potential to characterize and to quantify transgene activity spatially and noninvasively in absolute terms.

Materials and Methods

CD-UPRT–expressing tumors. Walker 256 cells were grown in minimal essential medium supplemented with 10% FCS. Cells transduced with a SFG retroviral vector (16) coding for CD-UPRT and neomycin resistance (Supplementary Fig. S3) were selected in the presence of 500 µg/mL geneticin (Invitrogen) and then a robustly CD-UPRT–expressing clone (validated through cellular accumulation of [3H]uracil) was propagated in antibiotic–containing medium. All tumors were inoculated by s.c. injection of 2 × 10⁶ W256 cells (wild-type or transduced) into the right flank of 5- to 6-wk-old male nude mice (nu/nu, National Cancer Institute). CD-UPRT gene expression was detected in tumors by immunohistochemistry as long as 7 wk after inoculation. Transplanted tumors were implanted under general anesthesia (ketamine/xylazine) and wound closure was achieved using a topical tissue adhesive (Nexaband Liquid, Abbott Animal Health). Tumor volumes and doubling times were determined as described previously (17).

To assay transgene expression in tumor tissue, Western blotting and immunohistochemical staining were performed as recommended by the manufacturer using a sheep anti–CD polyclonal antibody (Biotrend Chemicals, Inc.). The detection of apoptosis in tumor sections was achieved by immunohistochemical staining using the cleaved caspase-3 primary antibody according to the manufacturer's instructions (Cell Signaling Technology). Regional apoptosis was quantitated using MetaMorph software (Universal Imaging Corp.). For this purpose, regions of interest (ROI) were prescribed onto contiguous tumor sections (10 µm spacing) stained for cytosine deaminase gene expression. These ROI were overlaid...
Pharmacokinetic modeling. A five-compartment pharmacokinetic model was developed to simultaneously fit 2 one-compartment and 2 two-compartment models including 5-FU in plasma, 5-FU in both tumor types, as well as FNuc in each tumor type (Fig. 2A). The model is based on certain assumptions: (a) first-order kinetics for the cellular transport of 5-FU; (b) the enzyme-mediated conversion of 5-FU into FNuc and the further anabolism of FNuc are saturable and followed Michaelis-Menten constraints; (c) a single composite rate equation for the anabolism of FNuc. Certain kinetic variables were assumed to be the same for both the CD-UPRT− and CD-UPRT+ tumors, including (a) the rate constant describing the conversion of 5-FU into (k1) and out of (k2) the tissue; (b) the conversion of FNuc into anabolites not measured by MRS (Vmax,FNuc) and (c) the half saturation concentration for the 5-FU to FNuc reaction (Km,FNuc). The values for Vmax,FNuc and Vmax,FNuc were allowed to vary independently.

Each $^{19}$F-MRS tissue metabolite measurement was converted into a weighted average over five tumors using the inverse of the uncertainty as the weight. The uncertainty in each measurement was estimated to be at its CCR. The inverse of these uncertainties was later used during the fitting process to weight each data point. Metabolite concentrations that were below the detection limit were assumed to be at a median concentration of 200 μmol/L and were assigned an uncertainty of 600 μmol/L based on empirically determined thresholds (400 μmol/L) consistent with measured CRBs.

Liquid chromatography/mass spectrometry was used to measure plasma 5-FU concentrations drawn from non–tumor-bearing mice (n = 3 per time point) administered 150 mg/kg 5-FU. The resulting 5-FU plasma curve was used as a forcing function for the plasma 5-FU compartment. Each of the five compartments was associated with data from the $^{19}$F-MRS-derived time series. A nonlinear fitting procedure was used to achieve the best weight least-squares fit of each of the four tissue compartments to each of the four measured time series simultaneously. The half-saturation concentration for the UPRT-mediated reaction ($K_{m,FNuc}$) was fixed at 25 μmol/L (26). Five of the remaining variables ($k_1$, $k_2$, $V_{max,5FU}$, $V_{max,5FU}$, and $V_{max,FNuc}$) were allowed to vary during the iterative fitting procedure. Because of the potential that $K_{m,FNuc}$ would lack a clearly defined minimum, the fitting procedure was run repeatedly with $K_{m,FNuc}$ values ranging from 0.1 μmol/L to 1,680 μmol/L. Twenty-five fits were evaluated in total and the target function and variable values were noted (Supplementary Fig. S4A and B). Solutions to the rate equations and the nonlinear fitting were done using MATLAB v5.3 (Mathworks) routines.

Statistical analysis. Mean values are reported as means ± SE. Radiotracer accumulation was analyzed using the paired two-tailed Student’s t test. Apoptosis indices were analyzed using a one-way ANOVA.

Results
The CD-UPRT fusion enzyme was selected as a model transgene system for the imaging of transgene activity because of the versatility it offers with respect to MR-visible probes (Fig. 1). We initially compared 5-FC and 5-FU as potential probes for the assessment of CD-UPRT activity. Radionuclide uptake studies using $^{[13]}$C-5-FC or $^{[3]}$H-5-FU were done on wild-type Walker 256 (W256) carcinosarcoma cells (CD-UPRT−) as well as W256 cells stably expressing the CD-UPRT fusion gene (CD-UPRT+; Supplementary Fig. S1). These data, which show the accumulation of radiotracer at each time point in the modeled data. Corrections for intervoxel signal contamination were unnecessary for our voxel sizes (-3.0 mm × 3.0 mm × 5.0 mm) as described previously (25) and confirmed empirically (data not shown).

Figure 1. The CD-UPRT fusion enzyme can be monitored based on its metabolism of MR-visible probes to intracellularly entrapped MR-visible anabolites. Cells transduced with the yCDA-UPRT fusion gene (1) express the CD-UPRT enzyme (2). CD converts 5-FU to 5-FU, whereas UPRT anabolizes 5-FU to FUMP. FUMP and other fluorinated nucleotides are indistinguishable by MRS and appear as a single resonance on MR spectra that is designated as FNuc. As anions, these fluoronucleotides are unable to cross the cell membrane and are therefore maintained within the cell.
19F-MRS time series measurements (Supplementary Fig. S2) to a pharmacokinetic model describing the kinetics of 5-FU in CD-UPRT+ and CD-UPRT−/C0 tumors (Fig. 2A). Fits of the 19F-MRS–derived metabolite concentrations to this model (Fig. 2B and C) enabled the variables controlling the transport and metabolite conversions between the various compartments to be determined for wild-type and transduced tumors (Fig. 2D). The measured substrate concentrations (5-FU) greatly exceeded the reported UPRT $K_m$ (25 μmol/L; ref. 26) such that the UPRT was producing FNuc at or near to its maximal rate. As shown in Fig. 2D, the maximum rate of 5-FU anabolism in CD-UPRT+ tumors ($V_{\text{max,5FU}}^{\text{UPRT}}$) was 3-fold larger than that associated with the activity of endogenous enzymes in CD-UPRT−/C0 tumors ($V_{\text{max,5FU}}^{\text{wt}}$). The UPRT-specific $V_{\text{max,5FU}}^{\text{UPRT}}$ ($V_{\text{max,5FU}}^{\text{UPRT}} - V_{\text{max,5FU}}^{\text{wt}}$) was determined to be 1.113 μkat; this represents the maximum enzymatic rate specific to UPRT and, as such, is also a direct measure of the CD-UPRT protein concentration. Moreover, because the pharmacokinetic model follows Michaelis-Menten (nonlinear) kinetics, this UPRT-specific rate can be used to determine the activity of the CD-UPRT fusion enzyme for any single time point measurement of FNuc concentration.

This relationship allowed the application of the pharmacokinetic model to achieve quantitative images of transgene activity measuring regional CD-UPRT concentration and activity. Following the i.v. injection of 150 mg/kg (450 mg/m²) 5-FU into mice bearing CD-UPRT+ tumors, two-dimensional 19F-MRS images were acquired. These images were overlaid onto corresponding two-dimensional proton images to coregister the 19F-MRSI spectra with
tumor anatomy (Fig. 3). Each voxel of the $^{19}$F-MRS image shows the level of FNuc within a 0.045 cm$^3$ tumor volume with an in-plane resolution of 3.0 mm $\times$ 3.0 mm. The homogeneous delivery of 5-FU was indicated by the uniform distribution of fluorinated anesthetic observed throughout the tumor by MRSI (resonance located 75 ppm from 5-FU; data not shown). The measured FNuc concentration within these voxels enabled the generation of parametric maps of regional UPRT activity (Fig. 3A). In all but one of these tumors ($n = 8$), UPRT activity seemed to be homogeneous. In Fig. 3B, voxels I, II, and III show similar levels of UPRT activity ($\sim 0.841$ $\mu$kat) whereas UPRT activity in voxel IV is undetectable despite the presence of significant amounts of substrate 5-FU. In addition to the detection of heterogeneous enzyme activity within CD-UPRT$^+$ tumors, Fig. 3C shows that $^{19}$F-MRSI enabled the assessment of CD-UPRT concentrations and UPRT activity in mixed tumors constituted from equal parts wild-type and CD-UPRT–transduced W256 cells. As expected, the FNuc concentrations and the range of enzyme activity in mixed tumors is approximately half that of fully transduced tumors. This difference is further reflected in the decreased signal-to-noise ratio associated with the spectra in the mixed tumors compared with that in the fully transduced tumors.

Finally, we undertook experiments to investigate the relationship between MRSI-measured UPRT activity and the biological activity of this enzyme. For this purpose, we developed a mouse model in which sections of CD-UPRT$^+$ and CD-UPRT$^-$ tumors were transplanted side by side into the flanks of athymic nu/nu mice ($n = 6$). A representative example is shown in Fig. 4. Quantitative $^{19}$F-MRS images of these cotransplanted tumors showed a discreet pattern of regional UPRT activity (Fig. 4A). To correlate transgene activity, gene expression, and biological function, the acquired spectroscopic images were coregistered with tumor sections stained for CD-UPRT gene expression as well as caspase activity. As expected, tumor regions demonstrating UPRT activity by imaging correlated to those regions staining for CD-UPRT gene expression (Fig. 4B). Moreover, as early as 6 hours following 5-FU therapy, a significant difference in FNuc-induced apoptosis was observed in tumor regions exhibiting UPRT activity and expression compared with regions showing neither (Fig. 4C-E). Taken together, these data suggest that the imaging of transgene activity provides a relevant index of gene therapy.

**Discussion**

The studies presented here introduce a paradigm for the in vivo assessment of transgene activity through quantitative MRS imaging. The quantitation of in vivo metabolites in absolute units of concentration allowed the modeling of expressed transgene activity in absolute, SI coherent units of enzyme activity. In developing the pharmacokinetic model, we determined that a...
Michaelis-Menten analysis is required for single time point spectroscopic imaging of transgene activity (27). Using this analysis, we achieved the first reported quantitative images of transgene action in absolute terms and we were able to discern regional heterogeneities in the activity of the CD-UPRT fusion enzyme. MRSI enabled unique insights into regional heterogeneity that would not be discernible by other noninvasive imaging modalities. This point is underscored by the presence of a 5-FU resonance and the noticeable absence of a FNuc resonance in voxel IV of Fig. 3B. Assessments of this tumor using positron emission tomography would be unfeasible because signals resulting from 5-FU and FNuc would be indistinguishable, leading to the false impression of homogenous enzyme activity. In addition, we found that CD-UPRT concentration and activity were proportional to the percentage of CD-UPRT + cells. Furthermore, regional levels of UPRT activity as measured by imaging correlated with metabolite-induced apoptosis evincing that the noninvasive assessment of transgene activity can perhaps provide predictive insights into the therapeutic competency of a gene therapy system.

In this application, MRSI offers the potential for significant versatility as spectroscopic imaging of several MR-visible nuclei, including hydrogen, fluorine, phosphorus, and carbon (28), have been described. Transgene products that modify substrates containing these nuclei can potentially be monitored by MRSI reporter gene imaging. Several MRS reporter strategies have been developed, including creatine kinase, arginine kinase, and β-galactosidase (29–31). These strategies are not limited to intracellular proteins; MRSI has also been used to show extracellular enzyme function in vivo (32). This breadth of applications notwithstanding, several technical issues for the quantitative assessment of expressed transgenes by MRSI must be considered. The sensitivity of MRSI is inherently limited and sufficient metabolite concentrations must be sustained during image acquisition to achieve adequate spatial resolution. For example, the rapid anabolism of 5-FU by UPRT resulted in high concentrations of FNuc that were sustained for more than 5 hours in vivo. In contrast, the metabolism of 5-FC by CD-UPRT does not result in persistently elevated levels of fluorinated anabolites (13), suggesting that 5-FC would be a less effective probe for MRSI of CD-UPRT (data not shown). The anabolite levels required to image CD-UPRT activity in vivo are intrinsically related to the administered dose of 5-FU as well as the level of transgene expression. The described

![Figure 4.](image-url)
experiments involved a bolus i.v. injection of 150 mg/kg 5-FU, which represents the maximum tolerated dose of this agent in mice. Longitudinal studies of a single animal using this dosing regimen would be tolerated with a 10-day interval between 5-FU injections. The monitoring interval can be shortened by using lower doses of 5-FU. Our data indicate that the administration of as little as half of the maximum tolerated dose should be sufficient to achieve quantitative images of CD-UPRT activity. In many cases, even when metabolites are readily MR-visible in vivo, poor estimation of spin-lattice relaxation times in the tissue of interest may contribute to uncertainties in quantitation of tissue metabolite concentrations. We have previously reported the in vivo spin-lattice relaxation times of both 5-FU and FNuc in s.c. W256 tumors (12). In the setting of adequate substrate delivery, the sensitivity of our approach would be limited by the number of CD-UPRT+ cells. If we consider that a 1 cm³ tumor contains 10⁹ clonogenic cells (33) with an empirical ¹⁹F-MRCSI detection limit of 650 µmol/L for a 0.045 cm³ tumor volume (34), a conservative analysis of our data indicates that our approach should enable the detection of as few as 8 × 10⁶ CD-UPRT+ cells at 4.7 T. The delineation of transgene activity holds significant implications for the optimization of gene therapy in oncology as well as other disciplines. The correlation of expressed transgene levels and activity with dosing schedules and/or routes of administration could be used to evaluate the efficacy of vector delivery. Similarly, the ability of MRI to show differences in the activity of the expressed transgene could be applied to assess regional differences in vector distribution. Further, the accurate quantitation of tissue metabolites for the delineation of pharmacokinetic variables in absolute units could enable comparisons with conventional biochemical assays and facilitate the translation of in vitro data for in vivo application. For example, in the in vitro 5-FU IC⁵₀ of 1.04 × 10⁻⁸ mol/L, in combination with the in vitro [¹⁹F]FNuc accumulation rate of 0.35 mL·g⁻¹·min⁻¹ for CD-UPRT+ cells, indicates that UPRT enzyme producing a FNuc concentration of ~1.58 × 10⁻⁵ (mmol/L)·g⁻¹ will inhibit cell growth by 50%. The average FNuc concentration achieved in a single voxel of the imaged CD-UPRT+ tumor shown in Fig. 3A was 5.36 × 10⁻⁸ ± 3.10 × 10⁻⁴ (mmol/L)·g⁻¹. This intratumoral FNuc concentration was >300-fold higher than FNuc concentrations corresponding to the in vitro IC⁵₀ of 5-FU. The in vivo efficacy of this regimen is indicated by an associated CD-UPRT+ tumor doubling time of 13.9 ± 0.55 days compared with 1.8 ± 0.06 days for untreated CD-UPRT+ tumors. Indeed, the presented data suggest that the enzyme concentration and activity observed by in vivo MRI are therapeutically meaningful.

Importantly, our approach could be applied to acquire these data in a clinical setting. The described MR techniques are feasible using existing technology. Clinical MRI is routinely applied (35, 36) and quantitative MRIs of 5-FU pharmacokinetics in patients has been described previously (37) on available 1.5 T MR scanners. The current availability of higher field strength magnets for clinical studies (3-9 T) affords the opportunity for improved sensitivity in MRI. Ongoing clinical trials of 5-FC–CD cancer gene therapy (38) suggest that MRI monitoring of gene therapy could have immediate implications for the investigation of current strategies. Thus, MRI offers the potential to gain new and essential insights into in vivo transgene activity in both preclinical and clinical settings.

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References


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