An \(^{19}\)F Magnetic Resonance–Based \textit{In vivo} Assay of Solid Tumor Methotrexate Resistance: Proof of Principle

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ABSTRACT

\textbf{Purpose:} Studies in oncology have implicated multiple molecular mechanisms as contributors to intrinsic and acquired tumor resistance to antifolate therapy. Here we show the utility of an \(^{19}\)F-labeled methotrexate (FMTX) with \(^{19}\)F magnetic resonance to differentiate between sensitive and resistant tumors \textit{in vivo} and thus predict therapeutic response.

\textbf{Experimental Design:} Human sarcoma xenografts in nude mice were used in this study. The sarcoma cell lines chosen for this study (HT-1080, HS-16, and M-805) are well characterized in terms of their methotrexate sensitivity and molecular mechanisms of resistance. The pharmacokinetics of tumor uptake/washout of FMTX were monitored via \textit{in vivo} \(^{19}\)F magnetic resonance spectroscopy (pulse/acquire with surface coil localization) following an i.v. bolus injection. Response post-therapy, following leucovorin rescue, was monitored via tumor growth.

\textbf{Results:} The three tumor models show differences in both the peak concentrations of tumor FMTX and the dynamics of uptake/retention. These differences are most pronounced for time points late in the magnetic resonance observation period (225-279 minutes post-injection). A statistically significant linear correlation between tumor tissue concentrations of FMTX at these late time points and therapeutic response in the days/weeks post-treatment is shown (R = 0.81, F = 9.27, P < 0.001). Interestingly, a 400 mg/kg i.v. bolus injection of FMTX is a more potent cytotoxic agent \textit{in vivo} against methotrexate-sensitive tumors than is the parent compound (P = 0.011).

\textbf{Conclusions:} In principle, the assay method described herein could be implemented in the clinic as a diagnostic tool to make decisions regarding therapeutic protocol for the treatment of osteosarcoma on a case-by-case basis.

INTRODUCTION

Current standard of care for the patient with osteosarcoma involves an initial regimen of high-dose methotrexate for up to four cycles over the course of 10 weeks given in conjunction with cisplatin and doxorubicin. However, it is known that \(>50\%\) of osteosarcoma tumors in patients exhibit molecular evidence of methotrexate resistance (1, 2). Additionally, high-dose methotrexate therapy is not without potential complication. A diagnostic tool capable of predicting therapeutic efficacy at an early stage would be extremely useful in managing therapy in these patients. It is with these considerations in mind that we have undertaken the present work.

A number of factors at the cellular level contribute to methotrexate resistance in human cancers (1–5). These factors can include a failure of the cancer cell to transport the drug into the intracellular space or a failure to retain the drug intracellularly. The dianionic methotrexate molecule is primarily transported into the intracellular space via the reduced folate carrier (RFC). At very high extracellular methotrexate concentrations a small diffusional contribution has also been observed \textit{in vitro} (6).

The cytotoxicity of this drug is further potentiated via the action of the enzyme polyglutamate synthetase (FPGS). The FPGS enzyme conjugates multiple anionic glutamate residues to methotrexate, increasing intracellular retention. Decreased RFC activity is a common intrinsic methotrexate resistance mechanism in high-grade osteosarcoma in humans (2) with decreased FPGS activity representing a common mechanism of intrinsic resistance in soft tissue sarcoma (5). These and other means of resistance can also be acquired following initial treatment with high-dose methotrexate therapy (3, 4, 7).

The past, emphasis has been placed on monitoring plasma concentrations of methotrexate as a means to ensure therapeutic efficacy (8, 9). However, it is fundamentally important that any cytotoxic drug be delivered to the target tissue. We have developed an \(^{19}\)F-labeled methotrexate (FMTX, Fig. 1) with the goal of using \(^{19}\)F magnetic resonance (\(^{19}\)F MR) as a means to assay tumor methotrexate sensitivity. The synthesis of this pharmacokinetic probe has recently been reported (10). It has been noted that FMTX is readily MR visible \textit{in vivo} and its \textit{in vitro} cytotoxicity is equivalent to that of the parent antifolate (methotrexate) against a methotrexate-sensitive cell line.

Clinical \(^{19}\)F MR spectroscopy has been used previously to follow the tumor pharmacokinetics of 5-fluorouracil (5-FU) in a study of 57 patients, and it was shown that intratumor trapping of 5-FU is a positive predictor of therapeutic response (11). Subsequently, quantitative MR spectroscopy–based determinations...
of tissue concentrations of 5-FU and its metabolites in human liver were reported (12). In the case of a uniform excitation ($B_1$) over the volume of the sample, accurate quantitation requires knowledge of the $T_1$ relaxation times (or more precisely the ratio of $T_R/T_1$, where $T_R$ is the pulse sequence repetition time) of the metabolite and concentration reference species as well as the pulse angles experienced by the sample and reference (13). The more complex interrelationships among the factors affecting resonance intensity in the case of nonuniform excitation, as in the case of surface coil spectroscopy, have also been discussed (14). It has been emphasized that adequate signal-to-noise ratio is required for accurate determination of in vivo tissue metabolite concentrations (15). Variations and uncertainties in $T_1$ and/or MR pulse angle are potential sources of error. In the end, the choice of acquisition variables in MR spectroscopy is always a tradeoff between the need for acceptable signal-to-noise and trying to avoid quantitation errors (16).

Herein we address the utility of FMTX with $^{19}$F MR as a diagnostic tool in the early prediction of therapeutic outcome. Tumor tissue concentration can be monitored noninvasively in real time. The human tumor xenograft models studied in this work were the methotrexate-sensitive HT-1080 fibrosarcoma (5, 17); the HS-16 cell line, a mesenchymal chondrosarcoma, which is methotrexate resistant as a result of decreased FPGS activity (5, 17); and the malignant fibrohistiocytoma M-805, which exhibits resistance due to decreased RFC activity (18).

By studying the kinetics of drug uptake and retention in both sensitive and resistant human tumor xenograft models in mice, we show that it is possible to differentiate sensitive tumors from resistant tumors with this method. In addition to its potential clinical diagnostic utility, FMTX shows greater therapeutic efficacy in vivo than the parent compound, methotrexate.

Fig. 1 The chemical structure of FMTX and its potential metabolites. The FPGS reaction catalyzes the formation of FMTX-polyglutamates (FMTX-glun). DAMFPA and 7-OH-FMTX are formed by the action of carboxypeptidase and hydroxylase enzymes, respectively.
MATERIALS AND METHODS

Cell Lines and In vitro Cytotoxicity. The human sarcoma cell lines HT-1080 (5, 17), M-805 (18), and HS-16 (5, 17) have been described previously. The HT-1080 cell line was obtained from American Type Culture Collection (Rockville, MD) and the M-805 and HS-16 cell lines were obtained from the laboratory of Dr. Joseph R. Bertino (The Cancer Institute of New Jersey, New Brunswick, NJ). The HS-16 cell line is derived from a human mesenchymal chondrosarcoma, the HT-1080, a human fibrosarcoma, and the M-805 cell line from a human malignant fibrohistiocytoma.

Previously, it has been shown that the in vitro cytotoxicity of FMTX is equivalent to that of methotrexate against the HT-1080 cell line (10). In the current study, the in vitro cytotoxicities of methotrexate and FMTX were also compared against the HS-16 and M-805 cell lines. FMTX [N-(3-fluoro-4-amino-4-deoxy-N-methyl-pteroy1)-l-glutamic acid] was synthesized according to published methods (10) and methotrexate was obtained from Immunex (Seattle, WA). Cells were maintained as monolayer cultures in RPMI 1640 supplemented with 10% FCS at 37°C under a humidified 5% CO2 atmosphere. For cytotoxicity assays, monolayer cells were trypsinized and plated in 6-well culture plates (10 cm2 per well). After a period of 48 to 72 hours to allow for cell attachment and establishment of cell proliferation, the medium was aspirated and replaced with fresh medium and methotrexate or FMTX. Following a 24-hour drug exposure, culture medium was aspirated and replaced with fresh culture medium. Cells were incubated for a further 72 to 96 hours and cell viability was determined via the 2,3-bis[2-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt/phenazine methosulfate assay (19), 2,3-bis(2-Methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbony1]-2H-tetrazolium hydroxide and phenazine methosulfate were obtained from Sigma-Aldrich (St. Louis, MO). In vitro cytotoxicity was evaluated from drug concentration/response curves.

In vivo 19F Magnetic Resonance–Observed Tumor Pharmacokinetics. Animal studies were done according to institutionally approved protocols for the safe and humane treatment of animals. Tumor xenografts were initiated by the injection of 0.1 mL of a slurry of 106 cells. The tumor cell slurry was inoculated s.c. into the left flanks of 6-week-old male athymic nude mice (Charles River, Boston, MA). Tumors used in this study ranged in size between 0.16 and 0.41 cc. FMTX concentrations were estimated from the 19F MR intensity ratios using the measured longitudinal relaxation times (T1) for trifluoroacetic acid (3.37 seconds) and FMTX (0.60 seconds, from ref. 10) at 4.7 T and 37°C, the volume-averaged pulse angle applied to the tumor and external reference. This is similar to the method of Murphy-Boesch et al. (12, 23); however, in the current study, we do not generate a B1 field map in order to calculate precisely how the applied pulse angle varies across the region of interest. Tumor size and geometry were all roughly similar in the current set of experiments; thus, an empirically determined correction factor was measured to account for nonuniform excitation across the volume of the tumor. This volume-averaged correction factor was determined from a calibration curve generated from measurements on a series of spherical FMTX phantom samples (0.8 cm OD) of varying concentration using the 19F MR acquisition variables described above. The calibration curve (data not shown) relates the observed ratio of the FMTX and external reference 19F resonance intensities to FMTX concentration in the phantom sample. Tumor tissue volume average concentrations of FMTX are reported in millimoles/liter (mM).

Theoretical Modeling of 19F Magnetic Resonance Chemical Shift. Quantum chemical calculations were undertaken to obtain an estimate of the change in 19F magnetic shift with metabolism (see Fig. 1). The calculations were intended to ascertain whether or not the single observed in vivo 19F MR resonance arises from FMTX alone or whether some contribution could be due to FMTX-polylglutamates; the less potent FMTX metabolite, 7-hydroxy-FMTX (7-OH-FMTX, ref. 24); and/or the inactive metabolite, 2,4-diamino-methylfluoropteroic acid (DAMFPA, ref. 25). Theoretical calculations of the isotropic 19F MR chemical shifts for FMTX, 7-OH-FMTX, FMTX-glutamate, and DAMFPA were done using Gaussian 03 Revision C.02 software (Gaussian, Inc., Wallingford, CT). The molecular geometry was first optimized via a density functional B3LYP/6-31G* calculation, and the resulting molecular configuration was used in a restricted Hartree-Fock electronic structure calculation using the expanded 6-311+G** basis set (26). Calculations for each molecule were done on a PC with 2.4 GHz Pentium 4 CPU and required ~200 hours for completion. Isotopic 19F MR chemical shifts (in ppm) are reported relative to that for 19F-MTX.

Leucovorin Rescue and Tumor Response. At 6 hours post-FMTX administration, mice received i.p. hydration (1.0 cc normal saline) and at 24 hours received leucovorin (Bedford Laboratories, Bedford, OH) rescue therapy (27). This protocol allows the mice to survive what would otherwise be a fatal dosage of FMTX and is analogous to that used in high-dose methotrexate therapy in humans (28). Tumor growth was
monitored post-therapy. The three perpendicular axes of the tumor were measured with a micrometer and the tumor volume modeled as a spheroid \[ V = \left(\frac{\pi}{6}\right)abc \], where \( a, b, c \) are the dimensions of the tumor in cm. Tumor growth curves post-therapy were Gompertzian and hence in vivo surviving fraction (the fraction of cancer cells in the pretreatment tumor that survived therapy), SF, is reported based on the tumor volume growth variables:

\[
SF = \left(\frac{1}{2}\right)^{\frac{TGD}{DT_{Do}}} \tag{A}
\]

The variable \( DT_{Do} \) is the mean doubling time of the untreated, control group for each xenograft tumor model and TGD is the tumor growth delay for the treated tumor (the difference in tumor doubling time between the treated tumor and \( DT_{Do} \)). An assumption inherent in this model for determining SF is that the tumor growth rate is the same in the untreated tumor as in the post-treatment tumor during the regrowth phase, which is consistent with the observed tumor growth curves (see e.g., Fig. 2). For untreated growth the tumor volume doubling times \( DT_{Do} \) were 2.9 ± 0.1, 25.8 ± 5.5, and 23.4 ± 1.9 days for the HT-1080 \( (n = 8) \), HS-16 \( (n = 4) \), and M-805 \( (n = 6) \), respectively.

**Plasma Pharmacokinetics.** In a separate set of experiments, the time course of plasma pharmacokinetics was studied in mice. Each mouse received a 400 mg/kg i.v. bolus injection of FMTX. Plasma levels of FMTX were determined for the time points 0.5, 1, 2, 3, 4, 5, and 24 hours post-injection \( (n = 3-4 \text{ per time point}) \). Ten minutes before blood collection, mice were anesthetized with ketamine/xylazine. Blood was collected via cardiac puncture into heparinized vials and centrifuged. Typical collection volumes were 0.5 to 1 cc and hence this was a terminal procedure. To prepare the samples for high-performance liquid chromatography analysis 20 \( \mu \)L of 50% trichloroacetic acid was added to 200 \( \mu \)L of plasma. Samples were allowed to stand on ice for several hours after which the precipitate was removed by centrifugation and the supernatant collected for high-performance liquid chromatography analysis without further modification. An Agilent 1100 high-performance liquid chromatography system (Agilent, Palo Alto, CA) was used. Twenty microliters of the supernatant were injected onto an Econosphere C18 5 \( \mu \)m 4.6 × 250 mm column (Econosphere, Deerfield, IL) with an eluent of 15% acetonitrile/85% 50 mmol/L potassium dihydrogen phosphate at a flow rate of 1 mL/min and monitored at 313 nm. A standard curve of spiked plasma was linear from 0.31 to 10 \( \mu \)g/mL. Samples with higher drug concentrations were diluted to bring them to within the analytic range.

**Statistical Analysis.** The single factor two-sided ANOVA/Tukey test procedure (29) was done to check for statistically significant differences between mean values [including intratumor FMTX concentrations, area under the curve (AUC), and SF] for the three tumor models. Significance of linear regression was verified using the \( F \) test. Mean values are reported as mean ± SE unless otherwise indicated.

**RESULTS**

**Plasma \(^{19}\)F-Labeled Methotrexate.** In nude mice, plasma levels of FMTX follow biexponential decay kinetics after an i.v. bolus injection of a 400 mg/kg dosage (Fig. 3). The data were fit to Eq. B:

\[
[FMTX]_{\text{plasma}} = A \cdot e^{-k_1 \cdot t} + B \cdot e^{-k_2 \cdot t} \tag{B}
\]

The resulting fit variables were \( A = 0.480 \text{ mmol/L} \) with rate constant, \( k_1 = 1.74 \text{ h}^{-1} \) \( (t_{1/2} = 0.399 \text{ hour}) \) for the fast-washout component and \( B = 2.47 \times 10^{-3} \text{ mmol/L} \) and rate constant \( k_2 = 0.11 \text{ h}^{-1} \) \( (t_{1/2} = 6.3 \text{ hours}) \) for the second, slow-washout component \( (R = 0.91) \). The fit results in an estimate of \([FMTX]_{\text{plasma}} \) at \( t = 0 \text{ minute} \) of 0.482 mmol/L, whereas the value at the first measurement time point, 30 minutes post-injection was 0.32 ± 0.02 mmol/L. By 4 hours post-injection, plasma concentrations of FMTX fell to 1.7 ± 0.3 \( \mu \)mol/L and at 24 hours decreased further to 0.18 ± 0.09 \( \mu \)mol/L.

**\(^{19}\)F-Labeled Methotrexate Is more Potent than Methotrexate In vivo against a Methotrexate-Sensitive Tumor Xenograft.** Tumor growth curves indicate that FMTX is considerably more potent than methotrexate in vivo against the methotrexate-sensitive HT-1080 tumor xenograft model (Fig. 2). This is somewhat surprising because in vitro, both agents have equivalent cytotoxic action against both sensitive and resistant cell lines (data not shown). Analysis of the tumor growth post-therapy allows for a comparison of the efficacy of methotrexate and FMTX in terms of SF, the fraction of cells in the pretreatment tumor surviving therapy (Eq. A). The volume doubling time for the untreated HT-1080 tumor is 2.9 ± 0.1 days \( (n = 8) \). Following a 400 mg/kg i.v. bolus (FMTX or methotrexate), in the HT-1080 model, \( SF_{\text{FMTX}} = 0.29 \pm 0.06 \) \( (n = 7) \), whereas \( SF_{\text{methotrexate}} = 0.56 \pm 0.07 \) \( (n = 6) \), a statistically significant difference \( (P = 0.011) \). Even a dosage of 200 mg/kg FMTX was more potent than a 400 mg/kg methotrexate dosage, but not significantly \( (P = 0.051) \). At the 200 mg/kg FMTX dosage level, SF = 0.42 ± 0.04 \( (n = 5) \), whereas for 200 mg/kg methotrexate it was 0.64 ± 0.08 \( (n = 6) \).
Tumor Tissue Pharmacokinetics via 19F Magnetic Resonance. Tumor tissue concentrations of FMTX are readily MR visible with 18-minute temporal resolution following the 400 mg/kg i.v. bolus dosage (Fig. 4). In vivo tumor tissue pharmacokinetic data acquired by 19F MR show variation in drug uptake/retention between the xenograft models investigated in this study (Fig. 5). Theoretical quantum chemical calculations of the isotropic 19F MR chemical shifts for FMTX and its metabolites indicate that metabolized species would likely contribute to broadening of the observed in vivo tumor 19F MR resonance (Table 1). Separate signals from each metabolite would probably not be well resolved. The three tumor models (HT-1080, HS-16, and M-805) show differences both in the peak concentrations of tumor FMTX achieved and the dynamics of uptake/retention. The methotrexate-sensitive HT-1080 tumor model achieves peak tissue concentrations at 234 minutes post-injection (0.181 ± 0.054 mmol/L), whereas in the other two models, peak tumor concentrations are lower in magnitude and are achieved at earlier times preceding washout from the tumor tissue. In the M-805 tumor model, wherein, we would expect reduced uptake secondary to decreased RFC expression, peak tissue concentrations at 234 minutes post-injection (0.181 ± 0.054 mmol/L), whereas in the other two models, peak tumor concentrations are lower in magnitude and are achieved at earlier times preceding washout from the tumor tissue. In the M-805 tumor model, wherein, we would expect reduced uptake secondary to decreased RFC expression, peak tissue concentrations of FMTX occurs early (72 minutes post-injection) and are considerably lower (0.047 ± 0.021 mmol/L) than the maximum levels observed in the HS-16 and HT-1080 tumor models. The HS-16 tumor shows some ability to accumulate FMTX in the tumor tissue, but these levels peak at 126 minutes post-administration (0.077 ± 0.037 mmol/L) and are followed by drug efflux from the tumor as FMTX clears from the plasma, consistent with the FPGS-deficient status of this cell line. Although the mean maximum intratumor FMTX concentrations are greatest in the HT-1080 tumor, comparison of the maximum concentrations (HT-1080 at 234 minutes, M-805 at 72 minutes, and HS-16 at 126 minutes) does not reveal any statistically significant differences between methotrexate-sensitive and -resistant xenografts.

The differences in intratumor FMTX concentrations in sensitive (HT-1080) versus resistant (M-805 and HS-16) xenografts are most pronounced at later time points. Comparison of intratumor FMTX concentrations for the time point centered at 234 minutes post-injection (19F MR spectra acquired over the interval 225-243 minutes post-injection) indicate statistically significant differences between resistant and sensitive tumor models. Intratumor 19F-MR observable concentrations at this time point were 0.181 ± 0.054, 0.026 ± 0.020, and 0.025 ± 0.012 mmol/L for the HT-1080, HS-16, and M-805 tumor xenograft models, respectively. These concentrations are significantly higher for the HT-1080 than the M-805 (P < 0.001) and HS-16 (P < 0.001) tumors, whereas these last two groups do not differ significantly from each other.

Correlating Pharmacokinetic Variables with Therapeutic Efficacy. The tumor 19F MR kinetic data suggests the use of late time point intratumor FMTX concentrations as a means of differentiating between sensitive and resistant tumors. The AUC was estimated for the period from 225 to 279 minutes post-injection using the 19F MR intratumor concentration data (Fig. 5) and the trapezoidal rule. The calculated value is denoted as AUC225–279 (in mmol/L minutes). This quantity is significantly higher for the methotrexate-sensitive HT-1080 at 6.60 ± 1.42 mmol/L minutes than for either of the two resistant tumor models (P < 0.05). For the HS-16 and M-805 models, the values are 0.87 ± 0.60 and 0.75 ± 0.35 mmol/L minutes, respectively, and are not significantly different.

Plotting AUC225–279 versus log10(SF) yields a statistically significant linear correlation across the three tumor models investigated (Fig. 6). The resulting linear least squares fit to the full data set (n = 19) is:

\[
\log_{10}(SF) = -0.015 - 0.111 \cdot \text{AUC}_{225-279} \\
(R = 0.81, F = 9.27, P < 0.001).
\]

This fit very nearly passes through the origin (SF = 1 for AUC225–279 = 0 mmol/L minutes).
DISCUSSION

In oncology, MR imaging is routinely used in the clinical decision-making process. MR spectroscopy, on the other hand, is currently used primarily as a research tool, but is poised to play an ever-increasing role in the clinical setting (30–32). We note that $^{19}$F MR spectroscopic studies in humans have been reported for the antineoplastic agent 5-FU (11, 12, 33–35). Because they are typically localized to the extremities, osteosarcomas in the clinical setting are amenable to MR interrogation via surface coil with resulting benefits in terms of measurement sensitivity (36). The feasibility of obtaining well-defined MR spectra from bone tumors was initially unclear, but Nidecker et al. did $^{31}$P MR spectroscopic measurements of bone tumors (37) and later studies showed that $^{31}$P MR-observable tumor metabolic variables observed as little as 2 days post-therapy correlated with therapeutic response to chemotherapy (38).

Methotrexate is used in the treatment of many tumors, including acute leukemias, head and neck cancers, bone sarcomas, bladder tumors, and in some lymphoma patients. It is part of the standard chemotherapy regimen that has converted osteogenic sarcoma from an incurable tumor to its present status as a highly treatable and curable cancer. Thus, it remains in wide use despite the development of newer targeted agents and therefore a method to predict tumor sensitivity would have significant clinical importance.

Here we have shown that $^{19}$F MR spectroscopy of FMTX in vivo can be used to noninvasively differentiate between a methotrexate-sensitive tumor model (HT-1080) and two methotrexate-resistant xenografts with distinct mechanisms of resistance (HS-16 and M-805). The most pronounced differences occur at ~4 hours post-administration of an i.v. bolus of 400 mg/kg FMTX. The significant correlation between the area under the tissue concentration/time curve for the time points 225 to 279 minutes post-injection and the resulting therapeutic response indicates that this pharmacokinetic variable or some variation thereof may serve as a predictor of therapeutic efficacy.

Methotrexate is metabolized via a number of different pathways. The hydroxylation of methotrexate to form 7-OH-methotrexate occurs in the liver (39), whereas deactivation to the inactive 2,4-diaminomethylpteroyl acid (for which DAMFPA is the fluorine-containing analogue) results from metabolism of methotrexate by intestinal flora (40). As already mentioned, antifolate polyglutamates are formed in situ through the action of the FPGS enzyme. Theoretical quantum chemical calculations indicate that the FMTX metabolites, 7-OH-FMTX, DAMFPA, and FMTX-glu, if present, would probably lead to a broadening of the observed tumor $^{19}$F MR resonance. The predicted shift in the $^{19}$F resonance upon metabolism via these routes is of the order of 0.2 to 0.35 ppm (Table 1). Chemical calculations of the type we have employed are very reliable for the prediction of $^{13}$C, $^{15}$N, and $^{17}$O chemical shifts (26), but results for $^{19}$F should be approached with caution (41). However, in the case of fluorobenzenes (spanning a chemical shift range of ~63 ppm) the agreement between theory and experiment has been shown to be excellent (42). From a molecular modeling point of view FMTX is simply a complex fluorobenzene.

Because FMTX and its metabolites would likely not be well resolved in vivo $^{19}$F MR, it is necessary to consider the potential contributions of the metabolites to the MR resonance intensity, which is taken as an indicator of in situ cytotoxic potential. Specifically, we concern ourselves with the clinical scenario. A contribution by FMTX-polyglutamates to the $^{19}$F signal would be correctly interpreted as a positive predictor of therapeutic response, hence it is not problematic. The inactive metabolite 2,4-diaminomethylpteroyl acid is only occasionally detectable (~0.1μmol/L limit of detection) in the serum of patients following the administration of high-dose methotrexate therapy (43, 44). Thus, a spurious contribution of DAMFPA to the observed tumor $^{19}$F MR resonance is unlikely. However, plasma concentrations of 7-OH-methotrexate following high-dose MTX administration in humans are not negligible. This metabolite is cytotoxic, but considerably less so than the parent compound, methotrexate (39). The levels of plasma 7-OH-methotrexate show a marked dependence on the methotrexate dosage as well as the infusion protocol (43–45). In one study, immediately after a 6-hour infusion of high-dose methotrexate, the ratio of concentrations of methotrexate to 7-OH-methotrexate in plasma showed a range of ~10:1 to ~20:1 in patients (43). At later time points, with continued metabolism of methotrexate, the proportion of plasma antifolate species was increasingly skewed in favor of 7-OH-methotrexate. Thus, a contribution of the fluorine-containing analogue of this metabolite to the $^{19}$F MR resonance could be problematic at these later time points. Whereas we are uncertain about the levels of plasma 7-OH-FMTX in the current studies, it is worth reemphasizing that a statistically significant correlation between the tumor $^{19}$F MR signal intensity and therapeutic response was observed (Fig. 6) as described by Eq. C.

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Table 1  Theoretical $^{19}$F MR chemical shifts for FMTX and metabolites

<table>
<thead>
<tr>
<th>Chemical species</th>
<th>Predicted $^{19}$F MR chemical shift (ppm)*</th>
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<tbody>
<tr>
<td>FMTX</td>
<td>0.00</td>
</tr>
<tr>
<td>7-OH-FMTX</td>
<td>0.33</td>
</tr>
<tr>
<td>FMTX-glutamate</td>
<td>0.19</td>
</tr>
<tr>
<td>DAMFPA</td>
<td>0.36</td>
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*Chemical shifts are reported relative to the calculated chemical shift for FMTX.

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Fig. 5  FMTX tumor tissue pharmacokinetics as measured via $^{19}$F MR. Bars, ±SE for the last three time points.
The ability of HT-1080 tumors to steadily accumulate FMTX is remarkable. In this methotrexate-sensitive human sarcoma xenograft, tissue concentrations of FMTX reach a mean value of 0.18 mmol/L at 234 minutes, by which time plasma levels have fallen to \(~1.7\) mmol/L. Qualitatively, the shapes of the pharmacokinetic curves of model tumor FMTX uptake/retention can be understood in terms of the molecular mechanism of resistance. In the M-805 tumor model, decreased RFC activity leads to reduced tissue uptake, whereas in the HS-16 tumor, drug uptake is rapid with high tissue concentrations achieved but followed by rapid egress of FMTX from the tumor due to decreased FPGS activity. However, as we were unable to discern any statistically significant differences between the pharmacokinetics in the HS-16 and M-805 tumor models, it seems that the method outlined here may not be a definitive diagnostic of the resistance mechanism at the molecular level. The two tumor models, HS-16 and M-805, represent prevalent mechanisms of intrinsic resistance to methotrexate. There are other important molecular methotrexate resistance mechanisms, including active excretion pathways (3, 7); hence, FMTX pharmacokinetic behavior in these tumors might be expected to bear some resemblance to that of FPGS deficiency.

A surprising finding in this work has been the increased efficacy of FMTX, compared with methotrexate, against the methotrexate-sensitive HT-1080 tumor xenograft in vivo for the 400 mg/kg dosage. Molecular modeling indicates only slightly more favorable binding of FMTX in the active site of two of the key target enzymes, dihydrofolate reductase and thymidine synthetase (10). In vitro, the two agents are equipotent against the three cell lines investigated in this study. It is possible that the distinction in vivo arises as a result of differences in the rate of production of the less cytotoxic metabolite 7-OH-FMTX or the inactive DAMFPA, but we have not investigated this hypothesis.

The studies reported herein were done at 4.7 T, a magnetic field strength only modestly higher than the 3.0 and 4.0 T clinical MR systems currently being installed at many medical centers, suggesting the feasibility of performing similar studies on patients if biological data were compelling. Numerous studies have shown the clinical feasibility of monitoring pharmacokinetics in vivo via \(^19\)F MR. Reports that monitored

5-FU uptake, retention and metabolism in tumor and liver indicate that a temporal resolution on the order of 20 minutes per spectrum and a spatial resolution of 4 \(\times 4 \times 4\) cm is not an unreasonable expectation at 1.5 T (12, 34, 46, 47). As already noted, sarcomas are often superficial; thus, MR sensitivity would not be hampered by the tumor to detection coil distance. The approach we use of labeling a parent antineoplastic agent with one or even multiple \(^19\)F atoms (for increased sensitivity) may be applicable for monitoring the tumor pharmacokinetics of other drugs as well. Strategic positioning of the label atom could make it possible to monitor not just tissue concentration, but also metabolism in situ.

With regard to the feasibility of performing \(^19\)F MR with FMTX in humans, it is important to note that methotrexate is routinely given, not as a bolus, but as a 4- to 6-hour slow infusion (24); thus, the tumor tissue pharmacokinetics are likely to be different. Also, plasma levels of methotrexate in the range of 0.5 to 1.7 mmol/L (43) for a sustained period of several hours with these slow infusion protocols would likely lead to greater signal-to-noise in the MR measurement. For practical/financial reasons, it would not be possible to routinely follow the full drug uptake time course in tumors in the clinic. However, the data presented here suggest that the ability of methotrexate-sensitive tumors to concentrate and maintain elevated tissue levels of FMTX for longer periods may be a hallmark of therapeutic responsiveness to this antifolate. A single late time point \(^19\)F MR measurement, at or delayed from the conclusion of drug infusion, may be useful as a diagnostic test in the clinic. The results of such a test could potentially be used to direct the therapeutic strategy for each patient. A newly diagnosed osteosarcoma patient might be treated with methotrexate or trimetrexate based on the results of an initial FMTX \(^19\)F MR assay. Trimetrexate (24) is currently used in the treatment of patients with relapsed osteosarcoma because it can overcome methotrexate transport resistance, which is likely to be present in this setting. An early diagnosis (i.e., at the time of initial treatment) of a failure to accumulate \(^19\)FMTX within the tumor tissue could indicate the necessity of using trimetrexate at an earlier stage.

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An $^{19}$F Magnetic Resonance–Based In Vivo Assay of Solid Tumor Methotrexate Resistance: Proof of Principle


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