Quantified Tumor T1 Is a Generic Early-Response Imaging Biomarker for Chemotherapy Reflecting Cell Viability

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Abstract

Purpose: Identification of a generic response biomarker by comparison of chemotherapeutics with different action mechanisms on several noninvasive biomarkers in experimental tumor models.

Experimental Design: The spin-lattice relaxation time of water protons ($T_1$) was quantified using an inversion recovery-TrueFISP magnetic resonance imaging method in eight different experimental tumor models before and after treatment at several different time points with five different chemotherapeutics. Effects on $T_1$ were compared with other minimally invasive biomarkers including vascular parameters, apparent diffusion coefficient, and interstitial fluid pressure, and were correlated with efficacy at the endpoint and histologic parameters.

Results: In all cases, successful chemotherapy significantly lowered tumor $T_1$ compared with vehicle and the fractional change in $T_1$ ($\Delta T_1$) correlated with the eventual change in tumor size (range: $r^2 = 0.21$, $P < 0.05$ to $r^2 = 0.73$, $P < 0.0001$), except for models specifically resistant to that drug. In RIF-1 tumors, interstitial fluid pressure was decreased, but apparent diffusion coefficient and permeability increased in response to the microtubule stabilizer patupilone and 5-fluorouracil. Although $\Delta T_1$ was small (maximum of −20%), the variability was very low (5%) compared with other magnetic resonance imaging methods (24–48%). Analyses $ex vivo$ showed unchanged necrosis, increased apoptosis, and decreased %Ki67 and total choline, but only Ki67 and choline correlated with $\Delta T_1$. Correlation of Ki67 and $\Delta T_1$ were observed in other models using patupilone, paclitaxel, a VEGF-R inhibitor, and the mammalian target of rapamycin inhibitor everolimus.

Conclusions: These results suggest that a decrease in tumor $T_1$ reflects hypocellularity and is a generic marker of response. The speed and robustness of the method should facilitate its use in clinical trials. Clin Cancer Res; 16(1); 212–25. ©2010 AACR.

Chemotherapy remains a mainstay for the treatment of cancer that until 10 years ago involved predominantly cytotoxics but now also includes targeted agents. The successful development of the new generation of targeted agents and optimal application of existing agents including both cytostatics and cytotoxics requires the application of appropriate biomarkers. Biomarkers can serve several functions including attainment of the optimal biological dose (OBD), proof-of-concept, early markers of response, and stratification of the patient population. For all chemotherapies, but especially for the newer targeted agents, the OBD provides an alternative to the approach of using the maximum tolerated dose in phase I trials so that the effective dose is more rapidly identified and further trials can proceed using a less toxic but nevertheless effective dose and schedule. A recent example of this paradigm was seen in the development of the mammalian target of rapamycin inhibitor everolimus (1). Reliable early markers of response are relevant for all chemotherapeutics because (a) targeted agents tend to be cytostatic and may therefore not cause rapid changes in tumor size but can markedly increase stable-disease (2), and (b) in some indications, e.g., second-line ovarian cancer, response rates do not predict progression-free survival or overall survival even for cytotoxics (3). Stratification of patients based on the presence of specific molecular markers is particularly desirable, but may not always be possible and is still not routinely used (4), although here also generic markers could play a role.

Many generic biomarkers are now being used in the clinic to aid drug development of both traditional cytotoxics as well as the targeted agents. The approaches include monitoring blood biomarkers and functional imaging, e.g., dynamic contrast-enhanced magnetic resonance (MR) imaging (MRI), MR spectroscopy, and positron emission tomography as well as minimally invasive measurement of tumor interstitial fluid pressure (IFP).
Translational Relevance

Early biomarkers of tumor response to therapy are useful in optimizing existing treatments but also for development of new drugs, in particular in phase 1 for identification of an optimal biological dose. Using eight different experimental tumor models, we have applied noninvasive functional magnetic resonance imaging to quantify a parameter called T1, which is the spin-lattice relaxation time of water protons. In all cases, except in drug-resistant models, successful chemotherapy rapidly lowered tumor T1, and the fractional change in T1 was correlated with the eventual change in tumor size. Thus, a change in T1 was a marker of drug sensitivity. These observations were made in different models using patupilone, 5-fluorouracil, paclitaxel, the VEGF-R inhibitor PTK/ZK, and the mammalian target of rapamycin inhibitor everolimus. Analyses ex vivo suggested that a decrease in T1 reflects tumor hypocellularity and is thus a generic marker of tumor response that should be an important aid to oncology drug development.

These methods are a powerful means of repeatedly investigating the target tissue, e.g., tumor vasculature and hypoxia, metabolism, proliferation, and drug pharmacokinetics (5–8). All these methods have been used as early-response markers (5–7, 9), but sparser clinical data suggest they could also be used as stratifiers, e.g., IFP was identified as an independent prognostic indicator in cervical cancer (10) and high tumor vascularity (Ktrans) was associated with a better response to sorafenib in renal cancer (11). Nevertheless, these methods remain less than optimal because of one or more of the following: (a) complex and expensive chemistry, (b) low sensitivity, (c) nonquantitative, (d) require injections and modeling, (e) not applicable to all indications, and (f) add significant time to a protocol. Furthermore, a generic marker of response should always changes in the same direction, but this is not always the case (12).

Herein, we report a fast and simple but fully quantitative noninvasive MR marker that measures the spin-lattice relaxation of protons in water (T1) using rapid inversion recovery (IR) true fast imaging with steady-state precession (TrueFISP). The method has been reported for optimizing measurement of dynamic contrast–enhanced MRI vascular parameters (13), but it is not usually quantified. Earlier studies using a different method showed that the T1 of experimental tumors was greater than in normal tissue and was considered to reflect predominantly the extracellular space (14–17), with a larger interstitial compartment giving a longer (higher) T1. In untreated tumors, a low tumor T1 has been correlated to increased necrosis (16, 18), low water content (14–20), high levels of soluble protein (15), and slow proliferation (21). Animals treated with chemotherapy or radiotherapy showed increases (22–24) and decreases (15, 25) in tumor T1. However, in four different quantitative T1 studies in the clinic, only decreases were detected in response to successful therapy with radiation or cytotoxics (26–29). Using eight different experimental tumor models, we show that T1 can be measured easily with minimal variation and does not change with tumor size. Five anticancer drugs with different mechanisms-of-action caused a rapid decrease in T1, that often preceded and was strongly correlated with the change in tumor size suggesting that a fractional change in T1 (ΔT1) was a useful generic early-response marker. Furthermore, ΔT1 did not occur in tumors resistant to that drug, and ΔT1 was positively correlated with the proliferation markers Ki67 and choline, suggesting that ΔT1 indirectly reflected the number of viable cells in a solid tumor. These data suggest that our novel T1 method could be applied in the clinic as a generic marker of early response and for determination of an OBD.

Materials and Methods

All animal experiments were carried out strictly according to the local Swiss rules. Female Brown-Norway rats, C3H/He, and C57BL/6 mice were obtained from Charles River (France) and female Harlan Hsd:Npa nu/nu (nude) athymic mice were obtained from the Novartis breeding stock. Rats weighed from 135 to 180 g, whereas mice weighed 20 to 30 g before experiments.

Experimental tumor models

Eight different tumor models were used most of which have been previously described in detail. Murine RIF-1 fibrosarcoma were grown in the flank of C3H/He mice after injection of 5 × 10⁶ cells s.c. (30). Murine B16/BL6 melanoma cells (5 × 10⁵) were injected intradermally in the ear of C57/BL6 mice; these cells metastasise rapidly, especially to the lymph nodes of the neck (LN-mets), and these were the tumors studied (30). Rat mammary BN472 tumors were transplanted from donor rats by inoculation of fresh tumor material (25 mm³) in the mammary fat pad (31). The human tumor cell lines, HCT-116 colon, KB-31, and KB-8511 cervical cells were injected (2 × 10⁶) s.c. in the flanks of athymic nude mice (30, 32). The human cervical tumor cell line KB-8511 is an in vitro cholchicine-selected multidrug-resistant subline of KB31, which overexpresses P-gp and is thus cross-resistant to the taxane paclitaxel, whereas KB31 remains highly sensitive to the drug.

The isogenic human tumor non–small lung cancer cell lines A549 wild-type (A549wt) and the epothilone-B (patupilone)–resistant mutant A549.B40 were obtained from Dr. Susan B Horwitz (Albert Einstein College Medicine, New York, NY). Both cell lines were grown in RPMI 1640 containing 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, and 1% (v/v) l-glutamine at 37°C in 5% CO₂. A549.B40 is derived from A549 by stepwise selection with patupilone and contains a point mutation (Gln to Glu) that is associated with a 95-fold resistance to patupilone in vitro (33).
The A549.B40 cell line was grown and maintained in medium containing 10 nM patupilone. Both tumor types were created by s.c. injection of cells (2 × 10^6) in the flanks of athymic nude mice.

Except for the B16/BL6 LN-mets, which were measured by noninvasive MRI (see below), the size (volume) of all tumors (TVol) was determined using calipers to measure three orthogonal dimensions and applying the formula: \( V = \frac{4}{3} \pi r^3 \). TVol and animal body weight measurements were made thrice per week, just before treatment (baseline) and at the endpoint.

### Compounds/drugs and their application

All compounds and their respective vehicles were prepared each day just before administration to animals, and the administration volume was individually adjusted based on animal body weight.

Patupilone (epothilone B, EPO906) was obtained from the Novartis chemical department, Basel as a powder. It was dissolved in polyethylene glycol-300 (PEG-300) and then diluted with physiologic saline (0.9% w/v NaCl) to obtain a mixture of 30% (v/v) PEG-300 and 70% (v/v) 0.9% saline. Treatment with vehicle (PEG-300/saline) or patupilone was once weekly using an i.v. bolus in the tail vein (5 mL/kg for mice and 2 mL/kg for rats). Most of the experiments described lasted only 6 to 7 d, and thus, there was only one treatment in these cases. Doses were 1 to 5 mg/kg in mice and 0.75 mg/kg in rats.

5-Fluorouracil (5FU) was obtained as a powder from Sigma. 5FU (75 mg) was dissolved in 2.9 mL 0.9% saline. Treatment with vehicle (PEG-300/saline) or 5FU at a concentration of 25 mg/mL (pH 7). Mice were treated once i.p. (6 mL/kg) with 160 mg/kg 5FU or normal 0.9% saline.

Paclitaxel was obtained as a powder from Sigma and was dissolved in a vehicle of cremophor-ethanol (2:1 volume) before diluting 1:4 in 0.9% saline. It was injected as an i.v. bolus in the tail vein of mice thrice a week at 15 mg/kg (5 mL/kg), or just twice per week in the experiments lasting 4 d.

Everolimus (RAD001) was obtained from the Novartis chemical department, Basel as a microemulsion. The microemulsion was freshly diluted in a vehicle of 5% glucose and administered by oral gavage (p.o.) to mice daily (qd) in a volume 10 mL/kg at 10 mg/kg.

PTK/ZK (vatalanib) was obtained from the Novartis chemical department, Basel as a powder. It was dissolved in 100% v/v PEG-300 and then diluted 1:1 in 0.9% saline. Mice were dosed qd at 100 mg/kg p.o. (5 mL/kg).

### Experimental design

When tumors reached the desired size for the study of 100 to 500 mm^3 (normally circa 2 wk after cell inoculation), they were divided into different treatment groups and various measurements were made before treatment (baseline) on day 0 and at various time points after treatment. The posttreatment time points were selected as early (day 2 or 3) or late (day 6 or 7) with the latter normally being the endpoint, which was partly dictated by the size of the tumors in the vehicle-treated group. In some experiments, the endpoint was earlier (day 4) or later (day 14) and this is stated in the text and shown on the graphs. In many cases, after the final measurement at the endpoint, animals were sacrificed, the tumors ablated, and were studied by histology, immunohistochemistry (IHC), and/or ELISAs as described below.

### Magnetic resonance in vivo

Animals were anesthetized using 1.5% isoflurane (Abbott, Cham Switzerland) in a 1:2 mixture of O_2/N_2, and placed on an electrically warmed pad for cannulation of one tail vein using a 30-gauge needle attached to an infusion line of 30 cm to permit remote administration of the contrast agent (CA). The animals were positioned on a cradle in a supine position inside the 30-cm horizontal bore magnet and were anesthetized as above. Body temperature was maintained at 37 ± 1°C using a warm air flow and was monitored with a rectal probe.

All experiments were done on a Biospec DBX 47/30 spectrometer (Bruker Biospin) at 4.7 T equipped with a self-shielded 12 cm bore gradient system capable of switching 200 mT/m in 170 μs. Paravision Version-4 software for data acquisition and processing and 1H quadrature resonators with diameter of 35 or 70 mm (Rapid Biomedical GmbH) were used.

#### Quantitative T1 imaging

Tumor T1 was measured with an inversion recovery (IR) TrueFISP imaging sequence (13). The basic sequence was a series of 16 TrueFISP images acquired at a time interval, TI, of 210 ms after a global 180° inversion pulse. Each TrueFISP image (one transversal slice) was acquired with a flip angle α of 30°, a matrix size of 128 × 96, a field-of-view of 3 × 2.25 cm (mouse) or 6 × 4.5 cm (rats), a slice thickness of 2 mm, an echo time (TE) of 1.7 ms, and a repetition time (TR) of 3.4 ms. The time resolution for measuring the points on the recovery curve (i.e., the increment of TI) was given by the acquisition time of one image, i.e., 324 ms. The image series was started with a preparation pulse of flip angle α/2 that avoided signal oscillations in the transient state. The images were not averaged or segmented. Four image series with 0°, 90°, 180°, and 270° phase advance were acquired and averaged to eliminate possible banding artifacts that can be an inherent phenomenon of TrueFISP due to magnetic field inhomogeneities causing signal destruction through phase interference. Pixelwise T1 calculation of the image series was done off-line with home-written software based on IDL 6.0 programming environment (Research Systems, Inc.). A computing algorithm was used to find the correct polarity of the IR images acquired in magnitude mode (34). A three-parameter fit of the signal time course S(t) was done using the following model function (35):

\[
S(t) = S_{stat}\left[1 - ce^{-\frac{t}{TI}}\right]
\]

where \( t \) was the inversion delay TI.
Absolute T<sub>1</sub> was calculated from the flip angle \( \alpha \) of the TrueFISP sequence as follows (35):

\[
T_1 = T_1^0 \cos \frac{\alpha}{2} (c - 1)
\]

An example of the data obtained is shown in Supplementary Fig. S1. Tumor T<sub>1</sub> relaxation time was the mean of all tumor pixels of the T<sub>1</sub> map.

**Dynamic contrast-enhanced MRI.** Tumor T<sub>1</sub> was measured dynamically with the IR TrueFISP imaging sequence described above. The acquisition parameters were the same with the following exceptions: matrix size of 64 × 48, TI increment of 162.2 ms. The IR TrueFISP block was repeated 80 times with a temporal resolution of 8 s (10 s before CA infusion). The CA Vistarem (P792, Guerbet AG) was injected automatically at repetition #8 (0.8 mL/kg, which is 0.028 mmole/kg). Overall experimental duration was 12 min. As previously described (13), Vistarem was used for determination of the vascular constant for permeability \( K^\text{trans} \), leakage space \( V_L \), plasma volume \( V_p \) and initial (first 90 s) area under Vistarem enhancement uptake curve \( \text{iAUUEC} \). In one experiment, the CA Dotarem (GdDOTA, Guerbet) was used to determine the initial (first 90 s) area under Dotarem enhancement uptake curve \( \text{iAUUEC} \) following bolus injection of 0.2 mL/kg, which is 0.1 mmole/kg. The iAUUEC is a composite parameter reflecting both permeability and extracellular leakage space. We have found Dotarem and Vistarem to be equivalent for measurement of changes in iAUUEC.

**Dynamic susceptibility weighted MRI.** The CA Sinerem (Guerbet), an iron oxide nanoparticle intravascular CA, was injected i.v. (0.2 mmole/kg iron) for measurement of tumor vessel size imaging to obtain the blood vessel section as previously described (30) using the WIN method in C3H/He mice anesthetized with 2.5% isoflurane delivered at 2 L/min. IFP measurements were made before MR experiments.

**Histology**

Tumor slices were harvested from the largest circumference of the tumor, fixed in 4% phosphate-buffered formaldehyde for 24 h at 4°C and processed into paraffin as previously described (31). IHC was performed on paraffin sections as previously described (30) using the following antibodies for detection of (a) Caspase-3 (ASP175, Cell Signaling), (b) proliferating cell nuclear antigen (PCNA; ab29, Abcam), and (c) Ki67 antigen (ab833, Ki67 at 1:100; Abcam). For IHC, three to nine areas covering ∼10% of the total tumor area were captured at ×400 magnification in viable and nonhemorrhagic areas, and results were summarized as the percentage of positive staining cells for caspase-3, Ki67, or PCNA. In these viable regions, the total number of cell nuclei was used to provide an estimate of cell density. For necrosis, the total area was studied to provide the percentage-necrotic area.

**ELISAs for Ki67 and Tie-2**

Tumors were snap frozen and stored at −80°C until required. Tumors were homogenized in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 120 mmol/l NaCl, 1 mmol/l EDTA (pH 8), 6 mmol/l EGTA (pH 8.5), 1% NP40, 20 mmol/l NaF, 1 mmol/l phenylmethylsulfonyl fluoride, 0.1% SDS, 2 mmol/l sodium vanadate, and an anti-protease cocktail] using 1 mL per
100 mg tissue weight. Homogenates were kept on ice for 60 min and centrifuged for 15 min at 2,000 rpm at 4°C. The supernatant was passed through a 0.45-μm glass fiber filter and the filtrate stored at −80°C until further analysis. A kit (23227, Pierce) was used for the protein determination.

**Tie-2.** Tie-2 levels were measured as an *ex vivo* surrogate of tumor vascularity as previously described (39).

**Ki67.** The method described is an adaptation of that for *in vitro* samples (40). Plates (96-well) were coated over night at 4°C with 0.1 mL capture antibody anti-Ki67 (clone MB-1 or MM1) at 1 μg/mL. Wells were washed thrice with 0.2 mL TPBS (PBS/O Tween 0.05%) and blocked with 3% T-Block for 1 h at room temperature. Wells were then washed again thrice with TPBS, and protein lysates (0.25-2.5 mg/mL) in a total volume of 0.2 mL were added and shaken for 2 h at room temperature. MKI67 partial recombinant protein or Ki67 peptide (ab15581) dissolved in radioimmunoprecipitation assay buffer were used in a concentration range from 0.001 to 1,000 ng/mL in a total volume of 0.2 mL. After washing, a complex of detection antibody in TPBS (Mab anti-Ki67 clone SP6 or Pab anti-Ki67 clone ab15580 diluted 1:200, with alkaline phosphates conjugated to anti-rabbit IgG diluted 1:3,000) plus 0.1%Top-Block was applied for 1 h at room temperature. After washing thrice with TPBS and once with PBS/O, Ki67 antibody complexes were detected by incubating with paranitrophenyl phosphate and reading absorbance at 405 nm. The assay was validated from extracts of human KB-31 tumor cells by comparing Ki67 levels measured by the ELISA with those measured by a high-content fluorescence imaging platform that showed a strong positive correlation between the two different methods ($r^2 = 0.92, P = 0.002$).

**Data analysis**

Results are presented as mean ± SEM and all available data are shown except where stated. In some longitudinal experiments, data points are missing because of technical problems such as ineffective injection of a CA, or in rare cases, the model-fit required for a parameter was not possible (e.g., due to failed magnetization inversion because of field inhomogeneities). In one experiment described (Results), an outlier was determined based on the Grubbs test (extreme studentized deviate test; see Graphpad).3 The effect of a compound on a particular parameter, for example IFP, is summarized as the treated divided by control ratio ($T/C$; mean changes compared with baseline for drug-treated animals divided by the vehicle-treated animals) to provide a $T/C_{GFP}$. For TVol, body weight, and all *ex vivo* analyses, differences between groups were analyzed using a t test (for two groups) or a one-way ANOVA with Dunnett or Tukey tests post hoc. For the longitudinal BN472 experiment where *ex vivo* analyses were made on 2 separate days, a two-way ANOVA was also used. For the *in vivo* biomarker analyses, which involved longitudinal analyses in the same animals, differences were analyzed by (a) two-way repeated measures ANOVA and (b) t test or one-way ANOVA as appropriate for each time point; the latter method is therefore associated with the respective T/C. If necessary, data were normalized by a Log10 transformation before statistical analyses. Quantification of the *linear* relationship between fractional changes in $T_1$, expressed as the change in $T_1$ at the endpoint compared with baseline ($ΔT_{1_{\text{day-7/0}}}$) and other parameters were analyzed by Pearson’s correlation to provide the coefficient of determination ($r^2$) and the significance ($P$). Linear regression (which provides the same $r^2$ and $P$ values) was used to show the line of best fit and the 95% confidence limits. Correlations between basal $T_1$ and the endpoint tumor volume (dTVol) used data only from drug-treated animals. Note that these approaches assume that the data are normally distributed and that all relationships are linear, which may not always be the case. Corrections for multiple correlations in the same experiment at the same time point were made using the so-called "False Discovery Rate" or FDR (41). For all tests, the level of significance was set at $P < 0.05$ (two tailed) where $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)) versus vehicle.

**Results**

**Patupilone inhibits growth of murine RIF-1 fibrosarcomas and decreases tumor $T_1$**

Patupilone dose-dependently inhibited growth of RIF-1 tumors: $T/C_{\text{VOL}} = 0.37$, 0.09, and 0.02 for 3, 5, and 6 mg/kg, respectively (Fig. 1A). The basal tumor $T_1$ in untreated tumors ranged from 1.7 to 2.0 seconds with a very low coefficient of variation (CV) in each treatment group of just 2% to 4%. $T_1$ maps showed an even distribution across the central tumor slice with mean core values similar to those in the rim, although hotspots were present (Fig. 1B). Tumor $T_1$ in vehicle-treated mice was stable but was dose-dependently decreased by patupilone after 2 days, and this effect increased with time at the two higher doses giving a maximal decrease of 18% by day 7 (Fig. 1A). The lowest dose of patupilone had little or no effect on $T_1$, which may reflect that the TVol was already increasing at this dose on day 7. Furthermore, an earlier dose-response experiment gave a $T/C_{\text{VOL}}$ of 0.73 for 3 mg/kg (data not shown), suggesting that dose is really ineffective in this model.

Despite the relatively small changes in $T_1$, the fractional changes on day 7 ($ΔT_{1_{\text{day-7/0}}}$) were highly significantly positively correlated with the final change in tumor volume (dTVol) on day 7, and the relationship was stronger on day 2 (Fig. 1C). Thus, early changes in $T_1$ predicted the response of RIF-1 tumors to patupilone treatment. No significant correlation was apparent between basal $T_1$ values and dTVol day-7.

**Comparisons of patupilone-induced effects on RIF-1 tumor $T_1$, vascularity, and IFP**

To further investigate the nature of the drug-induced change in $T_1$, a single dose of 5 mg/kg patupilone on
various minimally invasive biomarkers was investigated at 2 and 6- days posttreatment and compared with histologic markers of apoptosis and proliferation at the endpoint.

RIF-1 tumor volume increased 3-fold over 6 days and this was blocked by patupilone (Fig. 2A). Basal tumor $T_1$ was similar to that in the first study (CV, 4%) and was significantly reduced by patupilone after 2 days and declined further by day 6 (Fig. 2A). Thus, although the magnitude of the maximum decrease in $T_1$ was relatively small compared with baseline ($-23\%$), the low variability meant the method was very sensitive for detecting a drug effect.

In contrast, other methods measured in the same mice had a much greater inherent variability. Vascular parameters measured by MR had a mean CV of 24% to 48%, but 85% for the vessel diameter (vessel size imaging), whereas IFP and the ADC had CVs of 43% and 34%, respectively. Patupilone tended to increase the ADC (Fig. 2B) and significantly increased the iAUEC (Fig. 2B). The iAUEC is a composite of permeability ($K_{\text{trans}}$) and $V_e$ (interstitial space), both of which also increased (Supplementary Fig. S3). IFP was significantly reduced by patupilone (Supplementary Fig. S3), but changes in other parameters reflecting tumor vascularity (blood or plasma volume, vessel size imaging) showed no

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![Graphs showing dose-dependent effects of patupilone on RIF-1 tumor growth and $T_1$. Murine RIF-1 fibrosarcomas were treated once by patupilone (3, 5, or 6 mg/kg i.v. bolus) or vehicle. Points, mean ($n = 8$) for tumor growth and $T_1$ (A) measured before and on days 2, 4 and 7 posttreatment with representative $T_1$ maps of tumors (2-mm slice) taken before and after treatment with vehicle or 5 mg/kg patupilone (B); bars, SEM. C, correlations between fractional changes in $T_1$ on day 7 or day 2 and changes in tumor volume (dTVol) 7 d after treatment. Note: one tumor (circled) was excluded from the analysis as an outlier by Grubb’s test.](image-url)
significant change. These data suggest that a decrease in T1 reflects increased extracellular space and/or permeability.

At the endpoint (day 6), histology and IHC showed no significant differences in necrosis, apoptosis, or PCNA between vehicle and patupilone-treated mice, but there was a significant decrease of 39% induced by patupilone in the %Ki67+ cells (Table 1; Fig. 2C) and a trend for the cell density (nuclei/mm²) to decrease by 12%. Parameters that had changed significantly following patupilone treatment were correlated with the fractional change in T1 (Supplementary Table S1). As in the first study, the endpoint dTVol was significantly correlated with both the early and late change in T1. However, there was no correlation between basal T1 values and the endpoint dTVol and none between the changes in T1 and iAUEC on either day 2 or day 5 (Supplementary Table S2). Some mice were culled for IHC on days 2 and 5 (n = 5 each day), which showed that 5FU induced a significant increase in the %apoptosis on day 5 (0.8 ± 0.4 to 2.6 ± 0.4).

5FU inhibition of RIF-1 growth is also associated with decreases in T1 and proliferation markers and increases in iAUEC

5FU strongly inhibited RIF-1 growth, causing regression on days 2 to 5, whereas vehicle-treated tumors grew rapidly and had to be culled already on day 5 (Fig. 3A). Drug-induced changes in T1 paralleled those for TVol so that significant decreases on day 2 to 5 were followed by a return to basal values by day 9 as tumor regrowth occurred. These changes were antiparalleled by the composite parameter of iAUEC, which suggested increased permeability and perfusion on days 2 to 5 (Fig. 3A). The endpoint dTVol (day 5) was significantly correlated with both the early and late change in T1. However, there was no correlation between basal T1 values and the endpoint dTVol and none between the changes in T1 and iAUEC on either day2 or day 5 (Supplementary Table S2).
and a significant decrease in the %PCNA+ cells on day 2 (92 ± 4 to 82 ± 2) and day 5 (93 ± 3 to 27 ± 5), whereas necrosis was not altered at either time point.

A second experiment of just 2 days confirmed that 5FU induced a significant decrease in T1 on day 2 (Table 1) and significantly increased by 3-fold the iAUEC. Necrosis was unchanged, but there was a significant increase in apoptosis of 3-fold and a significant 38-fold decrease in the %Ki67+ cells (Table 1; Fig. 3B). The ΔT1day-2/0 correlated significantly with %Ki67+ but not apoptosis (Supplementary Table S2).

In both 5FU-experiments, noninvasive 1H-MRS was done on some tumor-bearing mice to determine total tumor choline content (normalized as a fraction of the water signal) at baseline and 2 days after treatment (five vehicle and eight with 5FU). 5FU decreased Cho (T/CCho = 0.62, P = 0.05) and the change was significantly correlated with T1 or ΔT1 (Supplementary Table S2). There were insufficient tumors (n = 5) with IHC to correlate %Ki67+ cells with choline.

5FU-induced changes in RIF-1 tumor T1 are not paralleled by changes in MTR

As already shown in Figs. 1, 2, and 3, the T1 of untreated tumors was invariant, despite increases in TVol of >3-fold. Histology confirmed that during 2 weeks of RIF-1 tumor growth, there was no significant change in the %necrosis, %Ki67+ cells or %PCNA+ cells, nor in the noninvasive parameters of T1 and MTR, the latter measuring the ratio of bound to free water molecules (Supplementary Fig. S4). However, following the standard 5FU treatment, the T1 and proliferation parameters sharply decreased whereas necrosis and MTR were unchanged. These experiments were done in the absence of CAs, and a separate experiment confirmed that the CA Vistarem did not affect basal T1 or the ability of 5FU to reduce the T1 of RIF-1 tumors 2 days after treatment (Supplementary Fig. S5).

**Table 1. Summary of effects of different treatments on tumor growth, T1 and Ki67**

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatment, mg/kg</th>
<th>Endpoint day</th>
<th>T/C_TVol</th>
<th>T/C_T1</th>
<th>T/C_Ki67</th>
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</thead>
<tbody>
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<td>Murine RIF-1</td>
<td>PAT, 5</td>
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<td>0.81*</td>
<td>0.61†</td>
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<td>−0.19*</td>
<td>0.93†</td>
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</tr>
<tr>
<td>Human A549</td>
<td>PAT, 4</td>
<td>7</td>
<td>−0.01†</td>
<td>0.89†</td>
<td>0.67‡</td>
</tr>
<tr>
<td>Human A549.B40</td>
<td>PAT, 4</td>
<td>7</td>
<td>0.6</td>
<td>0.98</td>
<td>0.97</td>
</tr>
<tr>
<td>Rat BN472</td>
<td>PAT, 0.75</td>
<td>6</td>
<td>0.21†</td>
<td>0.90*</td>
<td>0.44‡</td>
</tr>
<tr>
<td></td>
<td>PTK/ZK, 100</td>
<td>6</td>
<td>0.72</td>
<td>0.92‡</td>
<td>0.72</td>
</tr>
</tbody>
</table>

NOTE: Results compare effects at the endpoint by the T/C for tumor volume, ΔT1, and Ki67 (measured by IHC or ELISA). The significance of the changes compared with the vehicle-treated group was tested by a two-tailed t test or 1-way ANOVA. For the BN472 model, a two-way ANOVA showed no significant effect for PTK/ZK on T1 but a significant effect for patupilone on Ki67. *P < 0.001. †P < 0.05. ‡P < 0.01. §P = 0.09. ¶P = 0.006.
affecting patupilone binding and shows strong resistance to patupilone in vitro: IC50 increased 80-fold [33]. Thus, patupilone dose-dependently inhibited growth of A549wt but was without significant effect on the A549.B40 mutant (Supplementary Fig. S7A; Table 1). Follow-up experiments showed that patupilone (4 mg/kg) significantly decreased the A549wt T1 and reduced Ki67 (Supplementary Fig. S7B). As in the other models, the patupilone-induced decrease in T1, but not basal T1, correlated significantly with the dTVol (Supplementary Table S3). The same dose of patupilone had no significant effect on the T1 or Ki67 of the mutant A549.B40 tumor (Table 1).

Thus, in two different isogenic tumor models, absence of a drug-induced change in T1 was associated with resistance to that drug and correlation with levels of Ki67.
Fig. 4. Effect of patupilone or paclitaxel on the growth, $T_1$, and Ki67 of KB-8511 and KB-31 xenografts. Human cervical KB-8511 (A and B) or KB-31 (C and D) tumors were treated by paclitaxel (15 mg/kg 3 once weekly i.v. bolus) or patupilone (4 mg/kg i.v. once) or patupilone vehicle once. Points, mean ($n=8$) on several different days posttreatment for the tumor volume and $T_1$ (A and C) and the endpoint Ki67 levels (ELISA; B and D); bars, SEM. Correlations are shown between the endpoint fractional change in $T_1$ and Ki67 (B and D).
Small effects on T1 associated with low basal Ki67 levels

Rat mammary BN472 tumors are sensitive to growth inhibition by patupilone (31) and the antiangiogenic PTK/ZK (42). The effects of patupilone, and as a comparator, PTK/ZK, were measured on tumor T1, caspase-3, and Ki67. Patupilone strongly inhibited growth, but PTK/ZK had little effect after 6 days of treatment (Table 1). Both PTK/ZK and patupilone had small effects on T1 compared with baseline of 2% and 4%, respectively, although these changes were significant compared with vehicle because of a significant increase in T1 in the vehicle group (Supplementary Fig. S8A). In this model, T1 changes did not correlate significantly with the endpoint dTVol (Supplementary Table S5). Importantly, IHC showed very low levels of Ki67+ cells in these tumors (0.4 ± 0.1). The Ki67-ELISA showed a trend for patupilone to reduce Ki67 protein (Table 1) and also the cell density as estimated from the total number of nuclei (Supplementary Fig. S8B). Patupilone highly significantly increased % Csp-3+ cells by 2-fold, whereas PTK/ZK was without any effect (Supplementary Fig. S8C). Despite the small changes in T1, the endpoint ΔT1 correlated significantly with levels of Ki67 protein and %Csp-3+ cells (Supplementary Table S4). Additional experiments using patupilone but without other biomarkers confirmed a minimal effect on T1 in this model.

Effects of everolimus and PTK/ZK on tumor T1

Murine B16/BL6 melanoma. Two compounds with different action mechanisms, the mammalian target of rapamycin inhibitor everolimus and the pan-VEGF-R inhibitor PTK/ZK, were tested for effects on T1 in two tumor models. Both compounds inhibited growth of the B16/BL6 mela-

oma lymph node metastases after 6 days of daily treatment, and this was associated with significant decreases in the T1 before significant changes in TVol (Fig. 5A and B). As in the other models, the ΔT1 correlated significantly with the endpoint dTVol whereas basal T1 did not (Supplementary Table S5). Furthermore, everolimus dose-dependently (1, 3, 10 mg/kg qd) reduced and patupilone (4 mg/kg once weekly) reduced the T1 of B16/BL6 tumors with positive correlations between the endpoint ΔT1 and dTVol (Supplementary Table S5).

Human HCT-116 xenografts. Everolimus, PTK/ZK, and patupilone inhibited growth of HCT-116 tumors (Supplementary Fig. S9A). The basal T1 was rather variable in the vehicle-treated mice during the treatment period, perhaps reflecting the increased heterogeneity of T1 in this model (CV, 7.5% at baseline). Despite this, all three compounds reduced the mean T1 significantly compared with vehicle from day 6 onwards (Supplementary Fig. S9A). In this model, there was a greater effect at the core compared with the rim, see for example PTK/ZK (Supplementary Fig. S9C). PTK/ZK caused the largest decrease in T1 and, consistent with that, the greatest reduction in Ki67 protein, too, which for all compounds correlated with the ΔT1 (Supplementary Table S6). As in the other models, the changes in T1 correlated significantly with the endpoint dTVol.

Discussion

We have shown that quantification of the change in the mean spin-lattice relaxation time of unbound protons in water (T1) in tumors provides a sensitive marker of the response to chemotherapy. Highly significant changes in T1 (ΔT1) were shown in several different models in response to drugs representing four different mechanisms of action,
and although $\Delta T_1$ was small (mean of 15-20%), the variability was low (c. 5%) compared with the other MR methods described here (24-48%) or that for FDG/FLT-PET (16-28%) using the same models (30). This high reproducibility meant that the TrueFISP method was very sensitive and suggested a $\Delta T_1$ threshold of $\geq 10\%$ signified a tumor response. In most cases, the $\Delta T_1$ preceded a significant change in tumor volume (dTVol) and was linearly correlated with dTVol at both early and late time points. It should be noted that these relationships may not always be linear, which may explain the variable $r^2$ observed, but the consistent significant correlation across all models suggests that $\Delta T_1$ is a reliable generic response marker. Indeed, isogenic models showed that tumors resistant to a specific drug failed to show a $\Delta T_1$, whereas the wild-type exhibited a drug-induced $\Delta T_1$. Furthermore, where studied, the effect was dose dependent. These results suggest that a drug-induced $\Delta T_1$ would be an excellent means of attaining an OBD in phase 1, and for later trials would be a useful early-response marker or maybe even stratifier after one treatment. Ultimately, these hypotheses have to be tested clinically.

What however does a drug-induced $\Delta T_1$ reflect or changes in the solid tumor? Previously, the $T_1$ of s.c. experimental tumors had been correlated to interstitial (extracellular) $H_2O$ content (14–16). These studies showed that the greater the amount of free water without macromolecules, the greater the relaxation time of the protons and, thus, the longer the $T_1$. Consequently, addition of soluble proteins dose-dependently decreased $T_1$ (15). Studies of untreated human melanoma xenografts showed that tumor $T_1$ was correlated positively with extracellular tumor water content (16, 18–20) and proliferation (21), but negatively with necrosis (18, 19). Theoretically, cellular destruction would lead to the release of macromolecules and denatured proteins, as well as paramagnetic ions from damaged erythrocytes and thus reduce $T_1$. Furthermore, a decrease in tumor blood volume/flow could reduce tumor $O_2$ content and consequently increase paramagnetic deoxyhemoglobin, which would also lower $T_1$ (43). On the other hand, drug-induced increases in tumor blood volume, flow, and permeability might increase the extracellular water space and thus raise $T_1$. Although we have not measured blood flow in this report, we did measure blood (BVol) and plasma (Vp) volume in RIF-1 tumors, and the endothelial marker Tie-2 in several models (data not shown), but found either no change or inconsistent changes, suggesting vessel density and blood volume did not reflect $T_1$. Solid tumors also have a significant inflammatory component (44), and these tumor-associated macrophages are influenced by the hypoxia and necrosis in a tumor and can also affect its angiogenic phenotype. Although successful chemotherapy can cause an influx of macrophages, this tends to be a late response and is therefore unlikely to influence the early changes in $T_1$ that we have observed (45). In previously described experimental tumor models, both decreases (15, 25) and increases (22–24) in $T_1$ have been reported in response to chemotherapy or radiotherapy. The conflicting data may reflect different methodologies, models, and the different time points measured, and extensive correlative histology was rarely performed.

We have observed in eight different models that increases in TVol of 3- to 10-fold have no impact on $T_1$, but successful chemotherapy leads to a significant decrease. In RIF-1 tumors, patupilone decreased $T_1$, and there were increases over the same time period in $K^{\text{trans}}$, interstitial space ($V_i$), and the composite of these two parameters, the iAUEC, as well as a strong trend for an increase in ADC, which reflects increased extracellular water content. IFP was also decreased, which could reflect decreased permeability, blood volume or cell number, or a composite of these (31, 42). However, because BVol and $V_p$ showed no consistent change and $K^{\text{trans}}$ was increased, it may be that in this model, the IFP decrease reflected overall cell death. In the same model, the cytotoxic 5FU also reduced $T_1$ and increased significantly the iAUEC. Importantly, there was no change in the MTR under conditions where 5FU reduced $T_1$. Although in principle the MTR and $T_1$ are both susceptible to macromolecular content, $T_1$ is more sensitive at detecting the free mobility of water, whereas MTR measures the exchange rate of bound with free water molecules (46). Consequently, in the RIF-1 tumor, the data obtained in situ supports the hypothesis that a decrease in $T_1$ reflected an increase in the extracellular space and/or overall permeability, in contrast to untreated tumors where an increase in extracellular space caused an increase in $T_1$ (16, 18–21). We suggest that increased permeability leads to a greater amount of serum in the extracellular space, and the increased tissue destruction to higher levels of macromolecules and paramagnetic ions, all of which would lower $T_1$. Neither 5FU nor patupilone affected necrosis, but apoptosis was increased in most cases, and the proliferation markers (PCNA and Ki67) increased, especially Ki67, which was significantly correlated with $\Delta T_1$. Furthermore, where measured, the cell density tended to decrease. Although Ki67 is considered as a proliferation marker, it is expressed in all phases of the cell cycle except G0 (47, 48) and can therefore be used as a surrogate of the number of viable cells in a growing tumor. Thus, in the RIF-1 model, we conclude that $\Delta T_1$ indirectly reflects a reduction in cell density and, consequently, an increase in extracellular space following cytotoxic therapy.

Such a comprehensive comparison of parameters was not performed in the other tumor models. However, it is likely of biological significance that in the model where very low basal levels of %Ki67 were measured (<1% in rat BN472 tumors), a drug-induced $\Delta T_1$ by patupilone or PTK/ZK was difficult to detect. We have found that basal levels of %Ki67 are much greater in other tumor models: 35% to 66% in murine RIF-1, 22% to 45% in human lung H-596, 43% in human breast BT474, and 70% in human colon HCT-116 (data shown here).4 These levels are more similar to those measured earlier.
in human tumors of 9% to 26% (48). Despite the low levels of Ki67 protein in BN472 tumors, there was a significant correlation of Ki67 with ΔT1. Furthermore, similar correlations between Ki67 and T1 were observed for both paclitaxel and A549, KB-31, and KB-8511 models, and also for everolimus and PTK/ZK in HCT-116 tumors. Apart from dTVol, Ki67 was the most consistent significant correlate with ΔT1, whereas changes in apoptosis and the endothelial marker Tie-2 either did not change significantly or did not correlate with ΔT1 in all models. Perhaps this is not surprising because the number of apoptotic and endothelial cells represents a small proportion of all cells (<5%) present in xenografts. It would be useful if basal T1 could be a prognostic marker of chemotherapeutic response, but we found no correlation between the endpoint dTVol and basal T1 in any model.

In conclusion, our data indicate that independent of the mechanism of action, successful chemotherapy causes a decrease in tumor T1, which most likely reflects the number of viable cells in a solid tumor. A decrease in the T1 of cancers has been shown following successful therapy with radiation and various types of cytotoxic chemotherapy (26–29), but has not been routinely applied. We believe that application of the IR TrueFISP method for measuring tumor T1 will provide a fully quantitative noninvasive method that does not require injection of a tracer or CA or complex modeling, yet provides a rapid read-out of cell viability in the target tissue. Thus, quantification of ΔT1 could be a major aid to cancer chemotherapy in the development of new drugs and better application of the current arsenal.

Disclosure of Potential Conflicts of Interest

All authors were employees of Novartis Pharma AG.

Acknowledgments

We thank Dr. Greg Burke for the inspiration and Dr. Terry O’Reilly for the encouragement, and Mike Becquet, Paolo Ferrara, Caroline Fux, and Christelle Gerard for their excellent technical assistance in animal handling and IHC. We also thank Guerbet AG for the supply of Vistarem and Sinerem.

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Received 3/19/09; revised 10/6/09; accepted 10/14/09; published OnlineFirst 12/15/09.

References


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