Changes in Pyruvate Metabolism Detected by Magnetic Resonance Imaging Are Linked to DNA Damage and Serve as a Sensor of Temozolomide Response in Glioblastoma Cells

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Abstract

Recent findings show that exposure to temozolomide (TMZ), a DNA-damaging drug used to treat glioblastoma (GBM), can suppress the conversion of pyruvate to lactate. To understand the mechanistic basis for this effect and its potential utility as a TMZ response biomarker, we compared the response of isogenic GBM cell populations differing only in expression of the DNA repair protein methyltransferase (MGMT), a TMZ-sensitivity determinant, after exposure to TMZ in vitro and in vivo. Hyperpolarized [1-13C]-pyruvate–based MRI was used to monitor temporal effects on pyruvate metabolism in parallel with DNA-damage responses and tumor cell growth. TMZ exposure decreased conversion of pyruvate to lactate only in MGMT-deficient cells. This effect coincided temporally with TMZ-induced increases in levels of the DNA-damage response protein pChk1. Changes in pyruvate to lactate conversion triggered by TMZ preceded tumor growth suppression and were not associated with changes in levels of NADH or lactate dehydrogenase activity in tumors. Instead, they were associated with a TMZ-induced decrease in the expression and activity of pyruvate kinase PKM2, a glycolytic enzyme that indirectly controls pyruvate metabolism. PKM2 silencing decreased PK activity, intracellular lactate levels, and conversion of pyruvate to lactate in the same manner as TMZ, and Chk1 silencing blocked the TMZ-induced decrease in PKM2 expression. Overall, our findings showed how TMZ-induced DNA damage is linked through PKM2 to changes in pyruvate metabolism, and how these changes can be exploited by MRI methods as an early sensor of TMZ therapeutic response. Cancer Res; 74(23); 7115–24. ©2014 AACR.

Introduction

Temozolomide (TMZ) is a chemotherapeutic DNA–methylating agent useful in the treatment of glioblastoma (GBM; refs. 1, 2). DNA O6-methylguanine (O6MG) lesions created by drug exposure mispair with thymine in the first cycle of DNA replication, leading to activation of the DNA mismatch repair system (3–5). The removal of the thymine opposite O6MG and subsequent reinsertion following repair resynthesis leads to cycles of futile mismatch repair. This in turn ultimately leads to DNA single-strand breaks (SSB), activation of sensors of DNA damage, the generation of DNA double-strand breaks (DSB), and delayed cell death by a combination of senescence and mitotic catastrophe (6–10). Because of its limited side effects and proven efficacy, TMZ is a component of the standard therapy for a variety of human brain tumors, and is frequently combined with other experimental agents (2).

Despite its ability to prolong disease-free survival, the response to TMZ-based therapies has proven difficult to monitor in patients. At present, standard imaging techniques are used to monitor the size of contrast-enhancing tumor following initiation of therapy, although for reasons related to difficulties in distinguishing tumor from necrosis and scarring, this approach often proves suboptimal (11, 12). Given that many patients, especially those with low-grade gliomas, can remain on TMZ for long periods of time (13), a more effective way to monitor drug efficacy and the early stages of drug failure in real-time would allow for better patient stratification as well as for the initiation of alternate approaches in cases of drug failure.

It has been known for more than 50 years that tumors metabolically differ from normal cells (14), an observation that has recently been applied to the noninvasive study of tumors by carbon-13 (13C) metabolic imaging. Tumors
frequently have high levels of lactate dehydrogenase A (LDH-
A; which preferentially converts pyruvate to lactate) and
NADH, the cofactor required for LDH-A activity, both of
which may explain the high levels of lactate found in many
tumors (15, 16). Changes in the expression of several other
tumor-specific metabolic enzymes have also been noted,
however, including a shift in expression from pyruvate
kinase (PK) M1 (which complexes with multiple glycolytic
enzymes and facilitates the shunting of pyruvate into the
citric acid cycle) to the less active M2 form of the enzyme,
which also may contribute to increased pyruvate to lactate
conversion (17–19). On the basis of these observations, the
administration of hyperpolarized compounds, most typi-
cally pyruvate, to tumor-bearing subjects, followed by the
real-time monitoring of conversion of the substrate to vari-
ous labeled metabolites using 13C 3D magnetic resonance
spectroscopic imaging (MRSI) should allow for the lo-
calization of malignant tissue (20). Studies along these lines
have proven promising, and further work suggested that the
approach could also be used to study drug response because
cells undergoing growth arrest or apoptosis convert less
hyperpolarized pyruvate to lactate (21–24). More recently,
the increased conversion of hyperpolarized pyruvate to lactate
was shown to precede the onset of tumor growth, and also
was reversed before tumor regression, at least in a myc-
driven murine model of liver cancer (25). These studies suggest
that hyperpolarized 13C MRI may be a promising way to
monitor tumor growth and perhaps tumor drug response.

We previously used hyperpolarized 13C MRSI monitoring
to show that human GBM xenografts exhibit an increase in
hyperpolarized pyruvate to lactate conversion relative to
normal brain (26, 27), and that TMZ exposure decreased this
conversion in a time frame that preceded histologic and
radiographic response (28). Although these findings sug-
gested that pyruvate metabolism might be linked to TMZ
response and could serve as a biomarker of TMZ action, it
was not clear whether the metabolic response was unique to
drug-sensitive cells, nor was the basis for the metabolic
change apparent. To better address these issues, we gener-
ate two pairs of isogenic GBM cells that differ only in
expression of O6MG DNA methyltransferase (MGMT), the
protein that removes TMZ-induced O6MG lesions before
they can mispair and lead to DNA damage, and as such
serve as the primary determinant of TMZ sensitivity (29–
31). Using these cells, we show that the TMZ-induced
suppression of the conversion of hyperpolarized pyruvate to
lactate is uniquely linked to the TMZ-induced DNA-
damage response via decreased PKM2 expression and intra-
cellular lactate levels, and as such serves as an early monitor
of TMZ response in brain tumor cells.

Materials and Methods

Cell culture

U87MG human GBM cells, and the same cells expressing
MGMT, P140K (U87 + MGMT), or siRNAs targeting PKM2
(U87 + shRNA PKM2) were obtained and cultured as described
previously (31, 32). Suppression of Chk1 protein (>80%) by
transfection of siRNA (Santa Cruz Biotechnology) was
verified by Western blotting. G55 is a human GBM cell line
passaged through nude mice and reestablished as a stable
xenograft (33), and was provided by the UCSF Brain Tumor
Research Center. The G55 cells form invasive intracranial
tumors in rodents that are more characteristic of primary
human GBM (33). The identity of both cell lines was verified
by DNA fingerprinting (Promega Powerplex 16) before initia-
tion of the studies.

Intracerebral tumor implantation and imaging study
scheme

Tumor cells were implanted intracerebrally into male
athymic rats (median weight 280 g) as described previously
(26–28). Animals received either oral vehicle (Ora-Plus; Pad-
dock Laboratories), oral TMZ (100 mg/kg), intraperitoneal O6-
benzylguanine (BG, 50 mg/kg), or BG followed 2 hours later
by TMZ when the tumor as monitored by T2-weighted axial
images was at least the size of one spectroscopic voxel
(approximately 86 mm3; typically 9–15 days after tumor cell
implantation). All animals underwent 13C and 1H imaging
before treatment (D0, baseline), and 1 (D1) and/or 2 (D2)
days following treatment.

Polarization procedure and 1H and 13C MR imaging

The polarization procedure and in vivo imaging have been
described previously (17, 24, 25, 27, 34–36). In brief, 13C imaging
data were acquired using a compressed sensing or fully sam-
poped 13C 3D MRSI sequence (24, 25) at 20 seconds from the start
of the injection of approximately 2.5 mL hyperpolarized
[1-13C]-pyruvate through the tail vein. The compressed sensing
data were acquired over 18 seconds, and the fully sampled data
over 17 seconds. The injection started approximately 10 sec-
onds after dissolution and lasted 10 seconds. For tumor volume
evaluation, T1-weighted anatomical images were obtained in
the axial plane after the injection of 0.1 mL Gadolinium (Gd)-
DTPA (approximately 0.2 mmol/kg; ref. 27).

Data analysis

The methods for processing 13C MRSI data have been
described previously (27, 36, 37). The 3D volume of the con-
trast-enhancing lesion was calculated from the axial T1 post-
Gd slices as described previously (36). At each time point a
percentage of tumor volume change from baseline was calcu-
lated, with the last time point being a composite of values
derived from multiple paired animals at the termination of the
experiment 3 to 7 days after TMZ exposure.

Immunohistochemistry

At D1 or D2 after drug treatment, animals were anesthetized
(1%–2% isoflurane) and perfused with 10% neutral-buffered
formalin (40 mL). The brains were then removed, fixed in
phosphate-buffered 10% formalin, dehydrated by graded etha-
nols, and embedded in Paraplast Plus wax (McCormick Sci-
entific). Tissue sections were incubated with rabbit monoclonal
phospho-Chk1 (Ser345) or polyclonal cleaved caspase-3
(Asp175) antibodies (Cell Signaling Technology) with antigen
retrieval. All immunohistochemistry assays were performed on

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the Benchmark XT (Ventana Medical Systems) using the UltraView detection system.

**Perfused cell studies**

Approximately 3 × 10^7 U87 or U87 + shRNA PKM2 cells were grown on Biosilon microcarrier beads (Nunc) for 48 hours, then loaded and analyzed in a 10 mm NMR tube connected to an NMR-compatible perfusion system, as previously described (23, 38).

**Protein extraction and immunoblot analyses**

Whole-cell lysates (30 μg protein) were subjected to Western blot analysis using antibodies against Chk1, MGMT, PKM2, and β-actin (all Cell Signaling Technology; ref. 32) with detection by a horseradish peroxidase–conjugated secondary IgG (Santa Cruz Biotechnology) using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

**LDH activity and NADH assays**

LDH activity and NADH levels were assessed from the D2 resected brains of control and TMZ-treated U87 tumor-bearing rats. Brains were rapidly removed after euthanasia, immediately snap-frozen in liquid N2, and stored at −80°C. For LDH activity, K_m and V_max values were determined by fitting initial rates of NADH consumption (absorbance at 340 nm) and pyruvate concentrations to a Lineweaver–Burke plot (39). Levels of NADH were determined in tumor extracts using a spectrophotometric enzymatic cycling assay kits (BioVision) according to the manufacturer’s instructions (40). In brief, tumor tissue was homogenized, centrifuged (14,000 rpm, 5 min) and heated (60°C, 30 minutes) to selectively decompose NAD^+ in the supernatant. The samples were then diluted 1:10 to fall in the linear range for a standard curve, and mixed with NADH reaction buffer. After 15 minutes of incubation at room temperature, changes in absorbance at 450 nm caused by the reduction of the reporter molecule thiazolyl blue were measured on a M200 Tecan spectrophotometer (Tecan Group) and compared with a standard curve established using known NADH concentration.

**PK activity and intracellular pyruvate and lactate measurement assay**

Intracellular PK activity, as well as concentrations of pyruvate and lactate were measured using PK activity, pyruvate, or lactate assay kits, respectively (BioVision).

**Results**

**In vivo biochemical and metabolic response of MGMT-modified GBM cells to TMZ**

To first determine whether TMZ-induced changes in pyruvate metabolism were related to drug response, two pairs of isogenic cells differing only in MGMT expression were used. TMZ-sensitive, MGMT-deficient GBM cells (U87 cells and G55 cells preincubated with the selective MGMT-depleting agent BG; ref. 41) exhibit increased levels of DNA SSB within 1 day of an IC_{50} TMZ exposure, accompanied by increased levels of the DNA-damage response protein pChk1, and the appearance of DNA DSB beginning 2 days after TMZ exposure (31). Isogenic TMZ-resistant, MGMT-proficient GBM cells (U87 cells expressing a construct encoding MGMT, and G55 cells in contrast, exhibit no increase in DNA SSB, pChk1, or DSB at 1 or 2 days following TMZ exposure (31).

In the first set of studies, control and MGMT-modified U87 GBM cells were intracranially implanted into rats and allowed to form tumors. Animals were then treated with vehicle or TMZ (100 mg/kg) and 24 or 48 hours later were administered [1,13C]-pyruvate and monitored for conversion of the substrate to lactate by 13C MRSI, for markers of the DNA-damage response, and for tumor growth. Nondrug-treated cells (U87 vehicle and U87 + MGMT vehicle) as well as the TMZ-resistant drug-treated U87 + MGMT + TMZ cells (31) exhibited an increase in pyruvate to lactate conversion both 1 and 2 days after TMZ treatment (Fig. 1A). The TMZ-sensitive drug-treated U87 cells (U87 + TMZ), however, exhibited decreased conversion of pyruvate to lactate (compared with pretreatment D0 tumors) beginning 1 day after TMZ exposure (Fig. 1A). The effect of TMZ on pyruvate to lactate conversion in these cells was consistent temporally with the appearance of cells that stained positively for activated pChk1 (Fig. 1B), but preceded the effect of the drug on tumor growth, which was not apparent 1 to 2 days after TMZ exposure in any cell group, but was apparent in the U87 + TMZ cells at the termination of the experiment 3 to 7 days after TMZ exposure (Fig. 1C; ref. 28). These results suggested that TMZ-induced changes in pyruvate to lactate conversion were related to the formation and/or response to TMZ-induced O6MG, and TMZ response.

To verify and expand on these results, MGMT-proficient, TMZ-resistant G55 GBM cells were intracranially implanted into rats, allowed to form tumors, treated with vehicle, the selective MGMT-depleting agent BG, TMZ only, or BG + TMZ, and similarly examined for effects on drug action and metabolic response. As with the tumors generated from the intracranially implanted U87 series of cells, nondrug-treated G55 cells (G55 + vehicle) as well as the G55 cells incubated with BG (G55 + BG) or TMZ alone (G55 + TMZ; ref. 31) exhibited an increase in pyruvate to lactate conversion both 1 and 2 days after TMZ treatment (Fig. 2A). The BG-treated, MGMT-depleted G55 cells (G55 + BG + TMZ), however, when exposed to TMZ, exhibited a decreased conversion of pyruvate to lactate (compared with pretreatment D0 tumors) beginning 1 day after TMZ exposure (Fig. 2A). The effect of TMZ on pyruvate to lactate conversion in these cells appeared coincident with the appearance of pChk1 staining at 1 day after TMZ exposure in the TMZ-sensitive G55 + BG tumors (Fig. 2B), but preceded the effect of the drug on tumor growth, which, consistent with the mechanism of action of the drug, was not apparent 1 to 2 days after TMZ exposure in any group (Fig. 2C), but was apparent in the G55 + BG + TMZ tumors, which at the termination of the experiment 3 to 7 days after TMZ exposure were 43% smaller than at 2 days, as opposed to G55 + TMZ tumors, which nearly doubled in size over the same time period. These results show that the effect of TMZ on pyruvate metabolism is directly related to the absence of MGMT, the generation of O6MG-related DNA damage, and/or the cellular response to these
lesions, all of which occur before the onset of TMZ-induced growth arrest and cell death.

The TMZ-induced metabolic response is not linked to inhibition of cell growth, induction of apoptosis, changes in NADH levels, or changes in LDH activity

Reductions in the conversion of $^{13}$C pyruvate to $^{13}$C lactate in vivo imaging studies of drug-treated tumors have been ascribed to a variety of factors, including loss of cellularity, apoptosis-induced PARP activation and depletion of NADH levels, decreased LDH-A expression, and loss of LDH activity. In the present study, the decrease in conversion of pyruvate to lactate in response to TMZ occurred within 1 day of TMZ exposure in vivo, long before any changes were noted in tumor growth and long before TMZ-induced O6MG lesions are converted to DNA mismatches and DNA DSB in these cells (31). Similarly, the effects of TMZ on pyruvate to lactate conversion were noted in drug-sensitive cells in the absence of any effect on apoptosis because control and TMZ-treated U87 tumors exhibited equally low levels of caspase-3 staining 2 days after TMZ exposure (Fig. 3A), consistent with the reported inability of these cells to undergo apoptosis in response to TMZ (6). Similarly, although TMZ-sensitive GBM cells could be expected to activate PARP following TMZ exposure in response to TMZ-induced DNA strand breaks (42), no changes in the levels of NADH (Fig. 3B) or LDH activity (Fig. 3C) were noted in U87 tumors 2 days after TMZ exposure as both the LDH $K_v$ and $V_{max}$ were not significantly different between control and TMZ-treated U87 tumor cells at the time (2 days after TMZ) at which significant differences in pyruvate to lactate conversion were noted (Fig. 1A). These results show that the TMZ-induced changes in pyruvate to lactate that are unique to TMZ-sensitive cells occur before the onset of growth arrest or cell death, and whereas related to TMZ-induced DNA damage and the damage response, appear unrelated to NADH levels and changes in LDH activity.

TMZ alters PKM2 expression and PK activity in a way that contributes to changes in pyruvate metabolism

PKM2 is overexpressed in GBM (32) and in its tetrameric active conformation physically associates with other glycolytic enzymes (hexokinase, glucose 6-phosphate dehydrogenases, glyceraldehyde 3-phosphate dehydrogenase) that are part of the complex that controls the conversion of glucose to pyruvate and the ultimate fate of pyruvate. PKM2 has also been shown to be downregulated in cells exhibiting alterations in pyruvate to lactate conversion (25), suggesting that its regulation may contribute to the TMZ-induced changes in pyruvate metabolism noted in this...
To address this possibility, we monitored the effects of TMZ on PKM2 expression, PK activity, and pyruvate metabolism in TMZ-sensitive and TMZ-resistant U87 and G55 cells. Exposure of MGMT-deficient U87 or G55 + BG cells, but not MGMT-proficient U87 + MGMT or G55 cells, to TMZ decreased PKM2 expression (Fig. 4A) as well as PK activity (Fig. 4B) in a time frame (1 day following TMZ exposure) consistent with the decrease in pyruvate to lactate metabolism beginning at D1, whereas all the other groups exhibited increased conversion of pyruvate to lactate (A, right). The effect of TMZ on $^{13}$C metabolism in the MGMT-depleted, drug-treated tumors (G55 + BG + TMZ) coincided with the appearance of pChk1 staining at 1 day after TMZ exposure (B; magnification, $\times 200$), but preceded drug response, which was not apparent at D1 or D2 post TMZ exposure but was apparent in the G55 + BG + TMZ group at the termination of the experiment 3 to 7 days after TMZ exposure (C). The numbers below the x-axis of the graphs in A and C indicate the numbers of animals for each group. The $^{13}$C spectra at baseline, D1 and D2 post TMZ exposure for A are provided in Supplementary Fig. S2; $^* P < 0.05$. 

Figure 2. TMZ-induced changes in hyperpolarized $^{13}$C pyruvate metabolism precede MGMT-regulated drug response in a G55 GBM human orthotopic xenograft model. A, left, representative hyperpolarized $^{13}$C MRSI data show $T_1$ post-Gd images and Lac/ Pyl overlay map of G55 tumor-bearing animals at D0, D1, and D2 following vehicle, BG, TMZ (100 mg/kg), or BG + TMZ treatment. The MGMT-deficient, TMZ-treated group (G55 + BG + TMZ) exhibited a decrease in pyruvate to lactate metabolism beginning at D1, whereas all the other groups exhibited increased conversion of pyruvate to lactate (A, right). The effect of TMZ on $^{13}$C metabolism in the MGMT-depleted, drug-treated tumors (G55 + BG + TMZ) coincided with the appearance of pChk1 staining at 1 day after TMZ exposure (B; magnification, $\times 200$), but preceded drug response, which was not apparent at D1 or D2 post TMZ exposure but was apparent in the G55 + BG + TMZ group at the termination of the experiment 3 to 7 days after TMZ exposure (C). The numbers below the x-axis of the graphs in A and C indicate the numbers of animals for each group. The $^{13}$C spectra at baseline, D1 and D2 post TMZ exposure for A are provided in Supplementary Fig. S2; $^* P < 0.05$. 

Figure 3. TMZ-induced metabolic response is not related to induction of apoptosis, changes in NADH levels, or changes in LDH activity. TMZ-treated or vehicle-treated animals with MGMT-deficient U87 tumors were euthanized at 2 days after the initiation of treatment and their brain analyzed for caspase-3 staining (A), NADH level (B), and LDH activity assay (C); $N = 8$ for U87 + TMZ and $N = 7$ for U87 + vehicle.
conversion in the same cells in vivo (Figs. 1A and 2A). These results suggest that the DNA-directed effects of TMZ on PKM expression may drive alterations in metabolism. To address this possibility more fully, we used two different shRNAs to suppress PKM2 expression in U87 cells and then monitored the effects on pyruvate metabolism. As shown in Fig. 4C, shRNA-mediated suppression of PKM2 expression resulted in a decrease in PK activity similar to that noted following TMZ exposure, as well as decreases in steady-state levels of both pyruvate and lactate in U87 and G55 cells (Supplementary Fig. S3; ref. 32). More importantly, the TMZ-sensitive U87 cells in which PKM2 levels and PK activity were suppressed also exhibited the same significant decrease in the in vitro conversion of hyperpolarized [1-13C]-pyruvate to lactate (63.5% ± 4.4% of U87 control, Student t test P < 0.01, n = 3; Fig. 4D and E), as was noted in TMZ-treated U87 cells. Similarly, in vitro levels of intracellular pyruvate and lactate were also significantly reduced in TMZ-treated U87 cells, and TMZ + BG-treated, MGMT-depleted G55 cells (Supplementary Fig. S4). These results suggest that the DNA-directed actions of TMZ lead to an early suppression of PKM2 and PK activity (U87 + shRNA PKM2) exhibited decreased conversion of 13C pyruvate to lactate (E; *, P < 0.001).

pChk1 contributes to the link between TMZ-induced DNA damage and PKM2 expression/activity

Because TMZ-induced changes in PKM2 expression and pyruvate metabolism were noted within 1 day of TMZ exposure in MGMT-deficient cells, we considered the possibility
that the DNA-damage response was linked to these metabolism-related changes. Furthermore, because the onset of Chk1 activation in drug-treated, TMZ-sensitive cells noted here and in previous studies (31) temporally matched that of the changes in metabolism, we examined the specific role of Chk1 in controlling this phenomenon. To do so, U87 and G55 cells were infected with siRNA constructs targeting Chk1, after which effects on PKM2 expression and PK activity were monitored. As shown in Fig. 5A, introduction of either of two different Chk1 siRNAs reduced levels of Chk1 by approximately 80% relative to scramble control cells in both cell lines. Introduction of scramble siRNA into U87 or G55 + BG cells did not significantly change PKM2 protein levels, whereas exposure of these cells to an IC50 concentration of TMZ significantly decreased levels of PKM2 (Fig. 5B and consistent with data in Fig. 4A). This degree of TMZ-induced PKM2 suppression was significantly reduced by Chk1 suppression (last column vs. adjacent two columns, Fig. 5B). Consistent with these observations, exposure of control or scramble siRNA U87 cells, or of G55 + BG or G55 + BG + scramble siRNA cells to TMZ significantly decreased PK activity, an effect that was also lessened by Chk1 suppression (Fig. 5C). These studies show that the Chk1 activation caused by TMZ-induced O6MG damage also

Figure 5. The DNA-damage response is linked to PKM2 expression and PK activity. A, introduction of siRNAs targeting Chk1 decreased levels of Chk1 protein in U87 and G55 cells. PKM2 protein expression (B) and PK activity (C) were significantly decreased 2 days following an IC50 TMZ exposure (100 μmol/L, 3 hours) in TMZ-sensitive U87 control and scramble siRNA cells (left), and G55 + BG plus scramble siRNA cells (right), an effect that was significantly reduced by introduction of Chk1 siRNA into U87 cells and G55 + BG cells; N = 3; *, P < 0.05. D, schematic representation of the data.
Table 1. Effect of PKM2 knockdown on TMZ sensitivity

<table>
<thead>
<tr>
<th>Cell group</th>
<th>Colony number (% control)</th>
</tr>
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<tbody>
<tr>
<td>U87 control</td>
<td>100</td>
</tr>
<tr>
<td>U87 + shRNA PKM2</td>
<td>68 ± 7</td>
</tr>
<tr>
<td>U87 + PKM1</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>U87 + 100 μmol/L TMZ</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>U87 + shRNA PKM2 + 100 μmol/L TMZ</td>
<td>4 ± 2</td>
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<tr>
<td>U87 + PKM1 + 100 μmol/L TMZ</td>
<td>2 ± 1</td>
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</tbody>
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contributes to the decreases in PKM2 expression, PK activity, and intracellular lactate levels linked to changes in pyruvate metabolism.

**TMZ-induced changes in PKM2 expression and metabolism are a biomarker of response rather than a contributor to TMZ action**

Having shown that exposure of TMZ-sensitive, but not TMZ-resistant cells led to decreases in PKM2 expression and PK activity, and that these changes were sufficient to alter pyruvate metabolism, we also considered the possibility that these events were critical downstream components of the cytotoxic action of TMZ in addition to potential biomarkers of TMZ action. To address this possibility, we measured the cytotoxic potential of TMZ in drug-sensitive U87 cells and in the same cells in which levels of PKM2 were either genetically suppressed or in which PK activity was increased by virtue of overexpression of the highly active form of PK (PKM1). As shown in Table 1, whereas the suppression of PKM2 levels in TMZ-sensitive U87 cells that were shown to alter pyruvate metabolism modestly decreased the colony formation ability of the non TMZ-treated cells, it had no effect on TMZ-induced cytotoxicity. Similarly, although overexpression of PKM1 nearly doubled PK activity in U87 cells (32), it also had no significant effect on TMZ-induced suppression of clonagenicility. These results therefore suggest that, although TMZ-induced changes in pyruvate metabolism are linked to changes in PKM2 expression, PK activity, and ultimately TMZ-induced DNA adducts, these changes, although separable from the cytotoxic actions of the drug and not per se a contributor to the effects of TMZ on tumor cell clonagenicity, are a marker of TMZ activity (Fig. 5D).

**Discussion**

13C MRSI is capable of monitoring the growth and regression of oncogene-driven tumors (22), and can provide biomarkers of the response of solid tumors to DNA-damaging agents and radiation (21). Here, we show that 13C MRSI measurements of hyperpolarized pyruvate to lactate conversion, via linkage to regulation of PKM expression and DNA damage, may be useful in measuring the early response of glioma to the commonly used chemotherapeutic-methylation agent TMZ.

A variety of studies have used 13C MRSI to examine the linkage between tumor growth, regression, drug response, and pyruvate metabolism, with varying results. Rapid changes in pyruvate metabolism were related to consumption of NADH and subsequent inhibition of LDH-A associated with apoptosis or necrotic cell death (21). Additional studies in doxorubicin-treated breast adenocarcinomas, however, showed that drug-induced suppression of pyruvate to lactate conversion could be separated from the cell death process, although the metabolic changes were still linked to consumption of NADH and subsequent inhibition of LDH-A associated with drug DNA damage and PARP activation (43).

In the present study, the metabolic effects linked to TMZ sensitivity could be clearly separated from the cell death process and in turn linked to DNA damage, although the exact form of damage that triggers the change in metabolism noted can only be partially determined. The requirement for O6MG lesions, the timing of events, and the data from siRNA suppression studies, however, suggest that Chk1 activation may help link persistent DNA damage to the metabolic changes noted, thus making Chk1 important in both responding to DNA damage and in providing a noninvasively detectable signal of cellular response.

If O6MG-induced DNA damage and signaling lead to changes in cellular metabolism, how is this accomplished? The present studies suggest that PKM2, a key regulator of cell metabolism known to be overexpressed in GBM may be involved. TMZ-induced DNA damage and Chk1 activation led to decreases in PKM2 levels and activity. Because PKM2 is regulated by a variety of events, including phosphorylation, PKM2 may be a target of Chk1 and as such directly linked to the DNA-damage response (44–46). Alternatively, the relatively large number of Chk1 substrates may activate pathways that alter PKM2 localization as well as absolute levels in a less direct manner. A number of other kinases, including Chk2, are also activated by TMZ in the time frame consistent with the TMZ-induced metabolic changes, and this degree of redundancy may explain the only partial reposition of PKM2 levels and activity following Chk1 knockdown in TMZ-treated cells. The TMZ-induced decrease in PKM2 levels was in turn associated with decreased levels of pyruvate and lactate at least where measured in vitro. Given the previously described connection between reduced intracellular lactate levels and decreased conversion of 13Cpyruvate to 13C lactate (21), it seems reasonable that the TMZ-induced decreases in intracellular lactate levels noted here resulted in the decreased pyruvate to lactate conversion in TMZ-treated animals. Although direct proof of this linkage would require more complex methods such as diffusion-weighted spectroscopy to measure intracellular lactate levels in vivo (47, 48), the present data clearly show that PKM2 plays a key role in linking the DNA-damage response to monitorable changes in pyruvate metabolism that in turn relate to drug efficacy.

In addition to identifying the link between TMZ-induced DNA damage and pyruvate metabolism, and defining the
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mechanism by which this linkage occurs, the present study also has clinical implications. Current monitoring of the TMZ action relies on inaccurate and uncertain measures of tumor size gathered over long periods of time following drug exposure. The studies presented show that at least in a preclinical model of GBM, TMZ-induced changes in pyruvate metabolism can provide a much earlier and perhaps more reliable marker of tumor response. Detection of the TMZ-induced changes in metabolism do not rely on measurements of tumor size or contrast enhancement, and in fact appear to precede any drug-induced changes in these parameters. As such this approach may alleviate concerns about inaccuracies of tumor size measurements. In addition, TMZ-induced changes in metabolism are related to the same event (DNA damage) that leads to cell death, but do not appear to be part of the cell death process. As a result, direct drug-sensitivity information, rather than information indirectly inferred from predictors of DNA damage (such as MGMT promoter methylation; ref. 49), can be obtained rapidly, repeatedly, and in the actual tumor being treated within a day of drug exposure rather than days to weeks later. Clinically, this could allow more rapid decision making and could also limit the exposure of drug-insensitive tumors to mutagens such as TMZ. The latter point could be especially important in low-grade astrocytoma, which are frequently treated with TMZ for extended periods of time, and that can be driven to progression by TMZ-induced genetic alterations (50). Therefore, although metabolic changes may be driven by events directly linked to cell growth inhibition and cell death, the unique delayed action of TMZ may provide links between the DNA-damage response and cell metabolism that can be exploited for improved therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: I. Park, J. Mukherjee, M. Itu, S.J. Nelson, R.O. Pieper
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References
5. D’Atri S, Tentori L, Lacal PM, Graziani G, Pagani E, Benincasa E, et al. Involvement of the mismatch repair system to the G2 checkpoint and to resistance to mutagens such as TMZ. The latter point could be especially important in low-grade astrocytoma, which are frequently treated with TMZ for extended periods of time, and that can be driven to progression by TMZ-induced genetic alterations (50). Therefore, although metabolic changes may be driven by events directly linked to cell growth inhibition and cell death, the unique delayed action of TMZ may provide links between the DNA-damage response and cell metabolism that can be exploited for improved therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Ilwoo Park, Joydeep Mukherjee, Motokazu Ito, et al.


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