Metabolic Activation of Temozolomide Measured in Vivo Using Positron Emission Tomography

Azeem Saleem, Gavin D. Brown, Frank Brady, Eric O. Aboagye, Safiye Osman, Sajinder K. Luthra, Alex S. O. Ranicar, Cathryn S. Brock, Malcolm F. G. Stevens, Edward Newlands, Terry Jones, and Pat Price

Cancer Research United Kingdom Positron Emission Tomography Oncology Group, Imperial College London [A. S. O., G. D. B., S. E. O. A., C. S. B., P. P. J.] and Imaging Research Solutions Ltd. [F. B., S. O., S. K. L., A. S. O. R.], Hammersmith Hospital, London W12 0NN, United Kingdom; Medical Research Council Cyclotron Unit, Imperial College London, Cyclotron Building, Hammersmith Hospital, Du Cane Road, London W12 0HS, United Kingdom [T. J.]; Cancer Research Laboratories, School of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom [M. F. G. S.]; and Department of Medical Oncology, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, United Kingdom [E. N.]

ABSTRACT

The purpose of this research was to quantitate and confirm the mechanism of in vivo metabolic activation of temozolomide. The secondary aims were to evaluate the tumor, normal tissue, and plasma pharmacokinetics of temozolomide in vivo, and to determine whether such pharmacokinetics resulted in tumor targeting.

$[^{11}C]^{\text{TEM}}$ kinetics were studied in men using positron emission tomography (PET). It has been postulated that temozolomide undergoes decarboxylation and ring opening in the 3–4 position to produce the highly reactive methyldiazonium ion that alkylates DNA. To investigate this, a dual radiolabeling strategy, with $[^{11}C]^{\text{TEM}}$ radiolabeled separately to indicate this fact.

$[^{11}C]^{\text{TEM}}$ and $[^{11}C]^{\text{TEM}}_{\text{a}}$ with tumor (26.8%), gray matter (29.7%), and white matter (30.1%), with ring opening over 90 min was less in plasma (20.9%; $P < 0.05$) compared with surrounding brain tissue. This pH-dependent activation of temozolomide may provide an important basis of targeted therapy directed toward tumors (such as gliomas), which are known to have a higher pH compared with surrounding brain tissue.

PET is a highly sensitive and quantitative imaging technique by which in vivo drug pharmacokinetics can be assessed using tracer quantities of the drug labeled with positron-emitting radioisotopes (7, 8). In this study we have used PET to investigate the in vivo mechanism of action of temozolomide in men including investigation of any differential in chemical conversion of temozolomide in tumor compared with plasma and normal tissue. PET studies were conducted in patients with gliomas with $[^{11}C]$-labeled temozolomide using a dual labeling strategy. Paired PET studies were performed after injection of $[^{11}C]^{\text{TEM}}$ radiolabeled in two different positions i.e. at 4-carbonyl and 3-N-methyl positions of the molecule. We hypothesized that if the proposed mechanism of action was true, then ring opening of $[^{11}C]^{\text{TEM}}$ at the 3–4 position would lead to the formation of $[^{11}C]^{\text{CO}}_{\text{2}}$ with $[^{11}C]^{\text{TEM}}$ and whereas with $[^{3}N]$-N-methylradiolabeled temozolomide the label will ultimately be incorporated into DNA (Fig. 1). The $[^{11}C]^{\text{CO}}_{\text{2}}$ produced during the metabolism of $[^{11}C]^{\text{TEM}}$ was quantified in plasma and exhaled air. Because PET is unable to distinguish radiolabeled chemical species in vivo, the contribution of $[^{11}C]^{\text{CO}}_{\text{2}}$ to the tissue image was elucidated using spectral analysis, a data-led analytical method. This required an additional PET study after the injection of $[^{11}C]$ radiolabeled bicitronate (which rapidly equilibrates with $[^{11}C]^{\text{CO}}_{\text{2}}$ in blood aided by carboxy anhydrase in the body) to derive a relationship between

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2 To whom requests for reprints should be addressed, at Molecular Imaging Centre, Academic Department of Radiation Oncology, Christie NHS Trust Hospital, Wilmslow Road, Manchester M20 4BX, United Kingdom. Phone: 44-161-446-8003; Fax: 44-161-446-8111; E-mail: Anne.Mason@christie-tr.nwest.nhs.uk.

3 The abbreviations used are: DTIC, dacarbazine; MTIC, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; PET, positron emission tomography; TAC, time-versus-radioactivity curve; AUC$_{\text{time-versus-radioactivity}}$ area under the plasma time-versus-radioactivity curve between 0 and 90 min; IRF, impulse response function.
plasma and tissue $[^{14}\text{C}]\text{CO}_2$. Therefore, it was possible to deduce tissue pharmacokinetics of $[^{11}\text{C}]$temozolomide with both $[3-N,^{11}\text{C}]$temozolomide and $[4,^{11}\text{C}]$carbonyltemozolomide. Furthermore, we proposed that if the postulated mechanism of chemical breakdown were confirmed, then the proportion of radiolabeled temozolomide out of the total radiotracer in tissue with $[4,^{11}\text{C}]$carbonyltemozolomide administration would give an indication of the amount of chemical conversion of $[^{11}\text{C}]$temozolomide.

**MATERIALS AND METHODS**

**Patients.** Six patients (all male; 27–63 years of age) were studied. All had histologically proven gliomas and a WHO performance status of 0, 1, or 2. Mentally unstable patients and those who had previous radiotherapy within the last 4 weeks were excluded from the study. Patients gave written informed consent to take part in the study, which had approval from the Hammersmith Hospital Research Ethics Committee and the Administration of Radioactive Substances Advisory Committee, United Kingdom.

**Study Design and Procedure.** Each patient underwent two PET studies (A and B) after the injection of $^1\text{C}$ (half-life 20.1 min) radiolabeled temozolomide with a period of at least a week in between the study periods. In study period A, two PET scans were performed, one after an infusion of $^{13}\text{O}$ (half-life 2.1 min) -labeled water to define vasculature and to aid in the definition of regions of interest (10 min duration) and another scan (90 min) after the injection of $[3-N,^{11}\text{C}]$temozolomide. Period B also consisted of two PET scans, a PET scan after injection of $[^{11}\text{C}]\text{HCO}_3$ (60 min) to correct for the contribution of $[^{11}\text{C}]\text{CO}_2$ and another scan after the injection of $[4,^{11}\text{C}]$carbonyltemozolomide (90 min). The initial PET scan was performed in one of the two study periods followed by the other. Radiolabeled temozolomide and bicarbonate were injected as bolus injections over $\sim 30$ s, whereas radiolabeled water was given over 20 s.

All of the scans were conducted as an outpatient procedure at the MRC Cyclotron Unit at the Hammersmith Hospital. Before each scan an arterial cannula was inserted into the radial artery under local anesthesia for plasma sampling throughout the scan. A venous line was also inserted for administration of the radiotracers. Patients were positioned on the scanner using laser beams for alignment. PET scanning was performed on an ECAT 953 (three-dimension) scanner (CTI/Siemens, Knoxville, TN; Ref. 9). This scanner allows simultaneous data acquisition to form 31 trans-axial planes with axial field of view of 10.8 cm (average full width at half-maximum = 5.8 mm). To enable correction for attenuation of photons in the body, a transmission scan was performed using a rotating $^{60}\text{Ge}/^{127}\text{Gd}$ rod source before tracer injection.

**Radiochemical Synthesis.** $[3-N,^{11}\text{C}]$temozolomide was synthesized as reported previously (10, 11). Briefly, $[^{11}\text{C}]$methylisocyanate was synthesized by passing $[^{11}\text{C}]$iodomethane in nitrogen over silver cyanate. Reaction of $[^{11}\text{C}]$methylisocyanate with the diazo precursor (5-diazoimidazole-4-carboxamide) yielded $[3-N,^{11}\text{C}]$temozolomide. For the synthesis of $[4,^{11}\text{C}]$carbonyltemozolomide, $[^{11}\text{C}]$methylisocyanate was prepared from $[^{11}\text{C}]$phosgene. Reaction of $[^{11}\text{C}]$carbonyltemozolomide with the diazo precursor resulted in the production of $[4,^{11}\text{C}]$carbonyltemozolomide. $[^{11}\text{C}]$bicarbonate was prepared as $[^{11}\text{C}]$sodium bicarbonate by the reaction of aqueous sodium hydroxide with $[^{11}\text{C}]$carbon dioxide.

Tracer quantities of temozolomide (mean, 1.35 $\mu$g; range, 0.47–2.59 $\mu$g) were administered during each of the scans and were at least 1/50,000 of the therapeutic dose range (150–200 mg/m$^2$/day). The mean injected radioactivity of $[3-N,^{11}\text{C}]$temozolomide and $[4,^{11}\text{C}]$carbonyltemozolomide was 258 MBq (range, 181–352) and 172 MBq (range, 114–269), respectively. These were associated with mean specific activities of 28.4 GBq (range, 17.7–37.4) and 40.1 GBq (range, 24.4–73.0), for $[3-N,^{11}\text{C}]$temozolomide and $[4,^{11}\text{C}]$carbonyltemozolomide, respectively. Mean radiochemical purity of the injected $[^{11}\text{C}]$temozolomide was 97.8% (range, 90.6–100%). Similarly, the mean activity of $[^{11}\text{C}]$bicarbonate was 369 MBq (range, 351–378), with a radiochemical purity of 100%.

**Plasma Sampling.** Plasma sampling was performed simultaneously with PET data acquisition throughout the PET study, to enable comparison of tissue radioactivity with plasma input. Continuous sampling of arterial blood radioactivity concentrations was performed via the arterial cannula. In addition, discrete plasma samples were also obtained at 2.5, 5, 10, 20, 40, 60, 75, and 90 min to ascertain the contribution of parent $[^{11}\text{C}]$temozolomide and $[^{11}\text{C}]$labeled metabolites to total plasma radioactivity.

Besides plasma $[^{11}\text{C}]$temozolomide radioactivity, the contribution of $[^{11}\text{C}]$CO$_2$ and other $[^{11}\text{C}]$metabolites to blood radioactivity was also determined. Metabolite analysis of $[^{11}\text{C}]$CO$_2$ was performed as described previously (12). Briefly, an aliquot of blood was mixed in sodium hydroxide, which trapped all of the $[11\text{C}]$activity, including all of the $[^{11}\text{C}]$CO$_2$ ($C_{\text{total}}$), as soon as the sample was withdrawn. Isopropanol and hydrochloric acid were added to another aliquot of blood, sample mixed, and nitrogen bubbled through it to release the $[^{11}\text{C}]$CO$_2$. The activity remaining after the removal of $[^{11}\text{C}]$CO$_2$ is given as $C_{\text{rem}}$. From this the percentage of $[^{11}\text{C}]$CO$_2$ ($C_{\text{CO2}}$) was given as:

$$C_{\text{CO2}} = \frac{C_{\text{total}} - C_{\text{rem}}}{C_{\text{total}}} \times 100$$

A third aliquot of blood was taken from the same sample of blood for analysis of $[^{11}\text{C}]$temozolomide and other $[^{11}\text{C}]$metabolites. This was centrifuged to obtain plasma, which was acidified to prevent chemical decomposition of $[^{11}\text{C}]$temozolomide. The plasma sample was deproteinized by addition of ice-cold acetonitrile, centrifuged, and the supernatant concentrated by rotary evaporation. The residue was dissolved in high-performance liquid chromatography mobile phase, filtered, and the filtrate analyzed on a reverse-phase column C18 $\mu$-Bondapak column (300 $\times$ 7.8 mm; 10 $\mu$m) using a mobile phase of ammonium acetate (50 mm) and acetonitrile ($95.5\% \text{v/v} \text{ (pH 6.7)}$) at 3 ml/min. The eluate was monitored with UV absorbance at 323 nm and radioactivity. If the percentage of $[^{11}\text{C}]$temozolomide out of total activity in this sample was Cmz, then the percentage of $[^{11}\text{C}]$temozolomide in plasma (Cplmz) was given as:

$$C_{\text{plmz}} = \frac{100 - C_{\text{CO2}}}{C_{\text{mz}}} \times C_{\text{mz}}$$

**Plasma Pharmacokinetics.** Plasma radioactivity was initially corrected for physical decay, and $[^{11}\text{C}]$temozolomide radioactivity (kBq/ml) was derived from the fraction of $[^{11}\text{C}]$temozolomide in plasma at various time points. TACs for $[^{11}\text{C}]$temozolomide were generated and the AUC$_{0\text{–}90\text{min}}$ was calculated using the trapezoidal method. Systemic clearance between 0 and 90 min (C$_{\text{cl}}$) was estimated from the injected dose of $[^{11}\text{C}]$temozolomide (kBq) and the AUC$_{0\text{–}90\text{min}}$ (Dose/AUC$_{0\text{–}90\text{min}}$).

**PET Data Analysis.** Sinograms were corrected for attenuation, detector efficiency (scatter, randoms, and dead-time), and detector nonuniformity, and reconstructed into tomographic images using the standard filtered back-projection algorithm (13). PET image data were calibrated to kBq/ml. Regions of interest on normal brain (gray and white matter) and tumor were manually defined on the PET images, with the aid of $^{18}$O PET scans and/or computed tomography/magnetic resonance imaging films using the image-analysis soft-

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**Fig. 1. Mechanism of action of temozolomide.** It was postulated that radiolabel in the 3-N-methyl position of temozolomide would be retained ultimately by the alkylating species after ring opening of temozolomide. On the other hand the radiolabel in the 4-carbonyl position will get converted to $[^{11}\text{C}]$carbon dioxide, which can be detected in the exhaled air and plasma.
Correction for Tissue Metabolites. As PET tissue data are unable to distinguish between the radiolabeled chemical forms of a compound in vivo, a data-led analytical approach (15) was adopted to quantify the contribution of metabolites in the tissue data. Because it was envisaged that $[11\text{C}]\text{CO}_2$ would be the primary metabolite in this study, an additional scan was performed after injection of $[11\text{C}]\text{HCO}_3$ to account for the contribution of $[11\text{C}]\text{CO}_2$ to the tissue image. The rapid conversion and equilibration of $[11\text{C}]\text{HCO}_3$ to $[11\text{C}]\text{CO}_2$ by the action of carbonic anhydrase in plasma was the basis for using $[11\text{C}]\text{HCO}_3$ to account for the contribution of $[11\text{C}]\text{CO}_2$. A similar correction for the contribution of $[11\text{C}]\text{CO}_2$ to the tissue data has been validated previously by our group in the evaluation of $[11\text{C}]\text{thymidine}$ PET scans, where the principal metabolite is also $[11\text{C}]\text{CO}_2$ (16).

The contribution of $[11\text{C}]\text{CO}_2$ to the tissue data was corrected using spectral analysis (15), a general deconvolution technique applied previously in oncology studies (16–18). Briefly, the relationship between plasma and tissue $[11\text{C}]\text{CO}_2$ in the $[11\text{C}]\text{HCO}_3$ scan was derived and referred to as unit IRF.

Tissue $[11\text{C}]\text{CO}_2(t) = \text{IRF}(t) \otimes \text{Plasma}[11\text{C}]\text{CO}_2(t)$ (Bicarbonate scan)

This relationship [IRF(t)] between plasma and tissue $[11\text{C}]\text{CO}_2$ was used to derive the tissue contribution of $[11\text{C}]\text{CO}_2$, in the temozolomide scans:

 Plasma $[11\text{C}]\text{CO}_2 \otimes \text{IRF}(t) = \text{Tissue}[11\text{C}]\text{CO}_2(t)$ (Temozolomide scan)

From this the contribution of $[11\text{C}]\text{temozolomide}$ to the tissue image was derived as follows:

Tissue $[11\text{C}]\text{temozolomide}(t) = \text{Tissue}[11\text{C}]\text{total}(t) - \text{Tissue}[11\text{C}]\text{CO}_2(t)$

Derivation of Ring Opening over 90 Min. If the hypothetical mechanism of chemical breakdown of temozolomide was true, then the generation of $[11\text{C}]\text{CO}_2$ after administration of $[4-11\text{C-carbonyl}]\text{temozolomide}$ would be indicative of the ring opening of $[11\text{C}]\text{temozolomide}$, and the amount of $[11\text{C}]\text{CO}_2$ generated would enable quantification of the total ring opening in the body for the scan duration (90 min). Therefore, ring opening over 90 min was derived after administration of $[4-11\text{C-carbonyl}]\text{temozolomide}$ from the ratio of total $[11\text{C}]\text{temozolomide}$ activity to the total $[11\text{C}]\text{tracer}$ activity and expressed as percentage ring opening ($\times 100\%$).

Ring opening over 90 min $= 1 - \frac{\text{AUC}_{0\sim90}[11\text{C}]\text{temozolomide}}{\text{AUC}_{0\sim90}[11\text{C}]\text{total tracer}}$

Thus, if there was no ring opening, then all of the activity over 90 min in the $[4-11\text{C-carbonyl}]\text{temozolomide}$ scan would be from $[11\text{C}]\text{temozolomide}$. Using this method, ring opening over 90 min was calculated in plasma, normal tissue, and tumor. This method assumes that breakdown of temozolomide occurs only by ring opening at the 3–4 position, with decarbonylation resulting in the generation of $[11\text{C}]\text{CO}_2$ from the radiolabeled carbon atom in the C-4 position.

Sampling of Exhaled Air. Exhaled $[11\text{C}]\text{CO}_2$ was sampled for the full duration of the scan by a loose-fitting single-use nasal oxygen set (Portex, Hythe, United Kingdom), the stems of which were positioned in the nostrils of the patient, as described previously (19). Air was drawn from the nasal set through a plastic scintillation detector to which it was connected using a nonpulsatile air pump set at 1.5 liter/min. The scintillation counter was cross-calibrated against a high-pressure ion chamber for purposes of quality control and assurance. The air line was also sampled downstream of the detector through a capnograph at a rate of 0.25 liter/min. Patients were asked to breathe normally through their nose during the procedure.

Exhaled $[11\text{C}]\text{CO}_2$, that was sampled for the full scan duration was corrected for physical decay and normalized for the injected activity of the radioisotope. This allowed the determination of exhaled $[11\text{C}]\text{CO}_2$, per ml of exhaled air, and the TACs of exhaled $[11\text{C}]\text{CO}_2$ were plotted. For ease of comparison, the $[11\text{C}]\text{CO}_2$ activities were calculated and expressed in units of % injected dose/ml of exhaled air. From this the total amount of exhaled $[11\text{C}]\text{CO}_2$ in each ml of sampled air over 90 min was calculated for both labels of temozolomide and compared.

No attempt was made to collect all of the exhaled air, as this would be associated with considerable patient discomfort and logistical difficulties in the conduct of PET scanning.

Statistical Methods. Paired and unpaired plasma and tissue data were compared by Wilcoxon and Mann-Whitney $t$ tests, respectively. Summary statistics and statistical comparisons were generated using STATA version 5.0 (Stata Corporation, College Station, TX). $P < 0.05$ were considered significant.

RESULTS

All of the patients completed both scanning periods except subject 3, whose condition deteriorated before the second set of scans (period A). PET images for patient 1 were lost because of technical problems during the first set of scans (period A). However, plasma and exhalation data were available for analyses. Thus, 6 PET images were available for analyses from period B and 4 from period A.

Plasma Pharmacokinetics. After administration of $[11\text{C}]\text{temozolomide}$, $[11\text{C}]\text{CO}_2$ was identified in addition to $[11\text{C}]\text{temozolomide}$ in plasma. Plasma $[11\text{C}]\text{CO}_2$ was detected for all of the studies after injection of $[4-11\text{C-carbonyl}]\text{temozolomide}$ (Fig. 2b), and in two of the four studies after administration of $[3-N-11\text{C-methyl}]\text{temozolomide}$ (Fig. 2a). No other $11\text{C}$-labeled metabolites of $[11\text{C}]\text{temozolomide}$ were identified for both labeling positions. The contribution of $[11\text{C}]\text{temozolomide}$ to total plasma radioactivity was higher when temozolomide was radio labelled in the 3-N-methyl position. The mean (SE) percentage of contributions of $[11\text{C}]\text{temozolomide}$, $[11\text{C}]\text{CO}_2$, and unidentified $[11\text{C}]$metabolites to the total $[11\text{C}]$ plasma radioactivity at 10 min with $[3-N-11\text{C-methyl}]\text{temozolomide}$ were 92.88 (3.9), 123 (1.2), and 5.90 (2.8), respectively. In comparison, mean (SE) percentage contributions of $[11\text{C}]\text{temozolomide}$, $[11\text{C}]\text{CO}_2$, and unidentified $[11\text{C}]$metabolites to the total $[11\text{C}]$ plasma radioactivity at 10 min with $[4-11\text{C-carbonyl}]\text{temozolomide}$ were 85.75 (2.1), 12.3
(1.9), and 1.96 (0.9), respectively. Similarly, at 90 min the mean (SE) percentage of contributions of $[^{11}C]$temozolomide, $[^{11}C]$CO$_2$, and unidentified $^{11}$C metabolites to total plasma radioactivity were 91.97 (0.7), 4.16 (2.5), and 3.87 (2.2) with $[3-N-^{11}C$-methyl]temozolomide, and 65.68 (4.6), 27.72 (2.0), and 6.6 (5.3) with $[4-^{11}C$-carbonyl]temozolomide, respectively. It was noted that $[^{11}C]$CO$_2$ was still generated (albeit in small quantities) with $[3-N-^{11}C$-methyl]temozolomide.

Cl$_{0-90}$ of $[^{11}C]$temozolomide was unaffected by the position of the radiolabel on the temozolomide molecule ($P < 0.05$) with mean (SE) Cl$_{0-90}$ being 0.195 (0.01) liters/min/m$^2$ and 0.212 (0.01) liters/min/m$^2$ with $[3-N-^{11}C$-methyl]temozolomide and $[4-^{11}C$-carbonyl]temozolomide, respectively. The similar clearance values of both radiolabeled forms would suggest the lack of any inherent instability in either of the two radiolabeled molecules. Plasma pharmacokinetic parameters were not estimated to infinity, as $25\%$ extrapolation from C$_{last}$ was obtained in some instances.

Exhaled $[^{11}C]$CO$_2$. As with plasma data, $[^{11}C]$CO$_2$ was detected in exhaled air after the injection of both radiolabeled forms of temozolomide (Fig. 3). However, a larger proportion ($P < 0.05$) was detected after injection of $[4-{^{11}C}$-carbonyl]temozolomide. The total amount of exhaled $[^{11}C]$CO$_2$ per ml of exhaled air with $[4-^{11}C$-carbonyl]temozolomide [mean (SE)] was 1.18 (0.09) $\%$ of injected dose. In contrast, the amount of exhaled $[^{11}C]$CO$_2$ in each ml of exhaled air with $[3-N-^{11}C$-methyl]temozolomide and $[4-^{11}C$-carbonyl]temozolomide, respectively. The similar clearance values of both radiolabeled forms would suggest the lack of any inherent instability in either of the two radiolabeled molecules. Plasma pharmacokinetic parameters were not estimated to infinity, as $25\%$ extrapolation from C$_{last}$ was obtained in some instances.

Normal Tissue and Tumor Kinetics. Visual inspection of PET images revealed that there was uptake of radiotracer ($^{11}$C-labeled temozolomide and metabolites) in both normal tissue and tumor (Fig. 4). To determine whether there was any difference in radiotracer exposure between the two labels, the radiotracer AUC$_{0-90}$ min was calculated for normal tissue and tumor (Table I). There was no difference in radiotracer exposure between both radiolabeled forms of temozolomide for the individual tissues. The similar AUC values noted with both forms would indicate that the parent compound (temozolomide) and its metabolites have similar properties of uptake by the tissues. Of potential therapeutic importance was the greater radiotracer exposure in tumors compared with normal tissue with both radiotracers. This may be because of either specific tumor targeting by temozolomide and its metabolites or may just reflect an impaired blood-brain barrier in patients with brain tumors.

Spectral analytical methodology was used to correct for the contribution of $[^{11}C]$CO$_2$ (the major and only radiolabelled molecule identified) to the tissue images (15, 16). This is illustrated in Fig. 5. Fig. 5, a and b, illustrate the TAC in tumor and gray matter, respectively, for a subject after injection of $[3-N-^{11}C$-methyl]temozolomide. As $[^{11}C]$CO$_2$ was not identified in plasma, in this study, no corrections were made for metabolites. Therefore, the total radiotracer activity in this instance is indicative of tissue $[^{11}C]$temozolomide activity. Fig. 5, c and d, illustrate TACs in tumor and gray matter, respectively, in the same subject after injection of $[4-{^{11}C}$-carbonyl]temozolomide, where plasma $[^{11}C]$CO$_2$ contribution was substantial. The top plots in Fig. 5 (unbroken lines) represent the total tracer ($^{11}$C-labeled parent and metabolites) activity. From this the contribution of $[^{11}C]$CO$_2$ to the total tracer activity was deconvolved using spectral analysis. This contribution of $[^{11}C]$CO$_2$ to tissue activity is plotted as a $[^{11}C]$CO$_2$ TAC (Fig. 5, closed triangles). The contribution of $[^{11}C]$temozolomide was obtained by subtracting $[^{11}C]$CO$_2$ activity from the total
DISCUSSION

Temozolomide is an alkylating agent that is structurally related to DTIC, with both drugs requiring conversion to the cytotoxic intermediate MTIC for clinical activity. Preclinical and mechanistic studies predicted a potential clinical advantage for temozolomide based on the nonenzymatic, pH-dependent generation of MTIC, which optimally occurs at physiological pH (6, 20, 21). This potential therapeutic advantage has been confirmed by clinical studies that have demonstrated promising anticancer activity particularly in patients with central nervous system malignancies and melanoma (22, 23). In addition, low interpatient variability in temozolomide clearance and excellent oral bioavailability (3) indicate a significant therapeutic advantage for temozolomide over i.v. DTIC, which requires metabolic activation via cytochrome P450 enzymes. Although MTIC was originally proposed as the alkylating species, it is now understood that the highly reactive methanediazonium ion produced by the reaction of MTIC with water at physiological pH is responsible for the methylating effect of temozolomide (6).

The site at which the conversion of temozolomide to MTIC occurs has been the subject of much speculation. It was initially proposed that conversion occurs in the DNA, with runs of guanine residues representing an accessible nucleophilic and basic microenvironment, which would facilitate conversion of temozolomide to MTIC, possibly by an “activated” water molecule in the major groove of the DNA. The electronic and steric effects of runs of three or more guanines were thought to enhance the nucleophilicity resulting especially in the methylation of the middle guanine of the N-7 residue (6). This theory was supported by the observation that MTIC preferentially methylates the N-7 residue (24). Against this theory was the observation that temozolomide activity correlated with the O6-methylation, with tumor cell lines expressing high levels of O6-alkyl-DNA alkyltransferase repair protein being refractory to temozolomide treatment (25, 26). In

Fig. 5. Tissue TACs for tumor (a and c) and gray matter (b and d) after injection of [11C]temozolomide. Plots a and b are after injection of [3-N-11C-methyl]temozolomide, and plots c and d are after injection of [4-11C-carbonyl]temozolomide. As plasma [11C]CO₂ was not detected after [3-N-11C-methyl]temozolomide in this patient, total tracer TAC was not corrected for metabolites. TACs were corrected for the contribution of [11C]CO₂ after administration of [4-11C-carbonyl]temozolomide. Plots c and d represent the total tissue tracer TAC (unbroken line), tissue [11C]temozolomide TAC (■) and tissue [11C]CO₂ TAC (▲). Metabolite correction was performed using spectral analysis.


<table>
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<th>Tissue</th>
<th>AUC₀₋₉₀ min Mean (SE) m²/ml</th>
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<tr>
<td></td>
<td>[3-N-11C-methyl] temozolomide</td>
<td>[4-11C-carbonyl] temozolomide</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.134 (0.03)</td>
<td>0.136 (0.01)</td>
</tr>
<tr>
<td>Grey matter</td>
<td>0.107 (0.005)</td>
<td>0.110 (0.001)</td>
</tr>
<tr>
<td>White matter</td>
<td>0.074 (0.007)</td>
<td>0.081 (0.004)</td>
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Comparison between tissues demonstrated total [11C] tracer exposure in tumors was higher in tumor compared with normal tissue (P < 0.05). Similarly, [11C] temozolomide exposure was higher in tumors compared with normal tissue, reaching significance (P < 0.05) with [4-11C-carbonyl]temozolomide studies (n = 6), but not with [3-N-11C-methyl]temozolomide (n = 4). Comparison within tissues showed no differences in exposure to total tracer when temozolomide was injected labeled in either of the positions. In contrast, exposure to [11C] temozolomide was higher within tissues after [3-N-11C-methyl] temozolomide injections tending towards significant levels (P = 0.07 for all tissues).

Percentage Ring Opening. Ring opening of [11C] temozolomide over 90 min was calculated in plasma, normal tissue, and tumor (Table 2). The percentage ring opening in plasma over 90 min (20.89%) was less (P < 0.05) than that observed in tumor (26.78%), gray matter (29.70%), and white matter (30.13%). However, there were no differences in the amount of observed ring opening between tumor and normal tissues (P > 0.05).

Table 2 Percentage ring opening of [11C] temozolomide over 90 min

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mean (SE) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>26.78 (0.97)</td>
</tr>
<tr>
<td>Grey matter</td>
<td>29.70 (0.97)</td>
</tr>
<tr>
<td>White matter</td>
<td>30.13 (0.97)</td>
</tr>
</tbody>
</table>

Tumor and normal tissues (P were no differences in the amount of observed ring opening between gray matter (29.70%), and white matter (30.13%). However, there was less (P 0.07 in tumor, gray, and white matter).

addition, all of the alkylating agents were associated with N-7 methylation, which was a characteristic of DNA rather than cytotoxic agents. Proof against DNA-based conversion of temozolomide was additionally supported from molecular modeling studies, which showed that the conversion of temozolomide occurs in free solution under the influence of local pH and additional DNA footprinting studies that were unable to detect noncovalent associations between DNA and temozolomide (5). The current thinking is that ring opening of temozolomide is entirely pH dependent and is supported by studies that have demonstrated that plasma half-life of temozolomide is in accord with the observed half-life of temozolomide in phosphate buffer (pH 7.4) at 37°C; Refs. 2, 3, 6.

In this study, we have applied PET methodology to investigate the mechanism of breakdown and, hence, the therapeutic rational of the mechanism of action of temozolomide. Specifically, we investigated if the proposed mechanism of action of temozolomide could be demonstrated in vivo in man using a dual radiolabeling strategy. Our hypothesis was that if ring opening of temozolomide occurs in the 3–4 position, as proposed, then the radiolabel will be carried either by the alkylating species or by carbon dioxide, when temozolomide was radiolabelled in the 3-N-methyl or 4-carbonyl positions, respectively.

We found support for this hypothesis when we sampled a 5-fold higher amount of exhaled [11C]CO2 with [4-11C-carbonyl]temozolomide administration. In addition, significant concentration of plasma [11C]CO2 was detected with [4-11C-carbonyl]temozolomide in contrast to small quantities of [11C]CO2 detected in plasma in two of the four studies with [3-N-11C-methyl]temozolomide. Supporting evidence was provided by tissue data, where a decrease in tissue exposure (AUC0–90 min) to [11C]temozolomide was observed with [4-11C-carbonyl]temozolomide compared with [3-N-11C-methyl]temozolomide, indicating the loss of radiolabel as [11C]CO2. These data provide proof of principle of the postulated metabolic breakdown of temozolomide. However, it was also observed in these studies that [11C]CO2 (albeit small quantities) was still generated when [3-N-11C-methyl]temozolomide was administered.

The calculated clearance between 0 and 90 min of [11C]temozolomide was 0.2 liter/min/m2 was higher than that estimated with other clinical studies (0.1 liter/min/m2) and was in keeping with the shorter duration of plasma sampled with this study.

In tissues, equivalent uptake of the radiotracer was demonstrated in both normal tissues and tumor with both forms of temozolomide, implying the absence of any molecular entities, which were sequestered in the plasma. Of interest and of potential therapeutic value in the clinical efficacy of temozolomide was the higher tumor exposure (AUC0–90 min) seen with temozolomide and total radiotracer compared with surrounding normal tissue. The higher AUC0–90 min observed for [11C]temozolomide in tumors compared with white and gray matter may provide a beneficial therapeutic index that may account for the clinical efficacy of temozolomide seen in gliomas (23). It is worth noting that the higher AUC0–90 min seen in brain tumors compared with normal brain may be related to a compromised blood-brain barrier and, hence, higher delivery, rather than retention of temozolomide. In this method, the contribution of non-[11C]CO2 metabolites (plasma contribution of non-[11C]CO2 metabolites was similar with both radiolabelled forms) to tissue radioactivity was not modeled separately.

Having confirmed the mechanism of breakdown of temozolomide, we set out to answer whether there was a differential in ring opening among plasma, normal tissue, and tumor, which may represent a targeted method of drug action in the tumor. If such a differential existed this would lend some support to the original hypothesis on temozolomide metabolism, where it was proposed that metabolic conversion occurred in DNA (6). In this study, we found a higher percentage of ring opening at 90 min in tissues compared with plasma, indicative of targeted tissue action as opposed to plasma. However, there was no difference in ring opening between tumors and normal tissue, suggesting that ring opening of temozolomide was tissue-specific but not tumor-specific. The methodology used to determine ring opening in tissues has a potential drawback in that it reflects both tissue-specific decarboxylation and influx of [11C]temozolomide and [11C]CO2 from the blood stream. Nevertheless, the inference on the differential in ring opening between plasma and tissue may still be true (although to a varying degree), as a higher amount of ring opening was observed in tissue despite the contribution from plasma, wherein ring opening was less. As a concluding remark of caution, we would like to state that calculation of the tissue metabolite correction is dependent on the method of analysis, which in the past has proven to be robust (16).

In conclusion, we have used PET methodology to ascertain the in vivo mechanism of metabolic breakdown of temozolomide using tracer amounts of the drug. In addition, this study has provided important information on tumor and normal tissue pharmacokinetics, not available by other means.

REFERENCES


Table 2 Percentage ring opening of [11C]temozolomide for the full duration of the scan

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Plasma</th>
<th>Tumor</th>
<th>Grey matter</th>
<th>White matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.73</td>
<td>26.89</td>
<td>28.32</td>
<td>26.80</td>
</tr>
<tr>
<td>2</td>
<td>27.79</td>
<td>33.33</td>
<td>26.13</td>
<td>26.36</td>
</tr>
<tr>
<td>3</td>
<td>21.54</td>
<td>20.58</td>
<td>27.78</td>
<td>28.75</td>
</tr>
<tr>
<td>4</td>
<td>21.04</td>
<td>30.66</td>
<td>38.18</td>
<td>38.36</td>
</tr>
<tr>
<td>5</td>
<td>18.99</td>
<td>21.78</td>
<td>29.36</td>
<td>28.24</td>
</tr>
<tr>
<td>6</td>
<td>17.25</td>
<td>27.42</td>
<td>28.44</td>
<td>32.05</td>
</tr>
<tr>
<td>Mean</td>
<td>20.89</td>
<td>26.78</td>
<td>29.70</td>
<td>30.13</td>
</tr>
<tr>
<td>(SE)</td>
<td>(1.5)</td>
<td>(2.0)</td>
<td>(1.8)</td>
<td>(1.8)</td>
</tr>
</tbody>
</table>

This was calculated from the ratio of total [11C]tracer: [11C]temozolomide after injection of [4-11C-carbonyl] temozolomide. Ring opening was greater in tissues compared with plasma (P < 0.05). However, there was no tumor-specific ring opening, as no differences in percentage ring opening were observed between tumor and normal tissues.


Metabolic Activation of Temozolomide Measured in Vivo Using Positron Emission Tomography

Azeem Saleem, Gavin D. Brown, Frank Brady, et al.


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