Phase II trial of neoadjuvant temozolomide in resectable melanoma patients

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Received 21 October 2009; revised 12 November 2009 & revised 24 November 2009; accepted 25 November 2009

Background: We treated melanoma patients with temozolomide (TMZ) in the neoadjuvant setting and collected cryopreserved tumor samples before and after treatment. The primary objective was to determine whether the response proportion was higher than previously reported in widely metastatic patients. A secondary objective was to test the feasibility of obtaining adequate tissue before and after treatment for genetic testing.

Materials and methods: Chemotherapy-naive melanoma patients who were candidates for surgical resection were eligible. TMZ was administered orally at 75 mg/m²/day for 6 weeks of every 8-week cycle. Cycles were repeated until complete response (CR), progression, or stable disease (SD) for two cycles.

Results: Of 19 assessable patients, 2 had CRs and 1 had partial response. Four patients had SD; 12 progressed. Tumor O-6-methylguanine-DNA methyltransferase (MGMT) promoter was unmethylated in all nine patients analyzed including from the two CR patients. Pretreatment tumor microarray results were obtained in 16 of 19 patients.

Conclusions: The response proportion to TMZ in the neoadjuvant setting was 16%, not different than in the metastatic setting. Responses were seen even in tumors with a methylated MGMT promoter. Pretreatment cryopreserved tumor adequate for microarray analysis could be obtained in most, but not all, patients. Post-treatment tumor was unavailable in complete responders.

Key words: microarray, MGMT

introduction

Temozolomide (TMZ), like dacarbazine, is a prodrug of the active metabolite 3-methyl-(triazen-1-yl)imidazole-4-carboxamide and is commonly used in the management of advanced melanoma. Its potential advantages over dacarbazine include oral administration as well as penetration through the blood–brain barrier. As a single agent in the treatment of metastatic melanoma, TMZ is associated with an objective response rate of 12%–14% for both the monthly dosing regimen (200 mg/m²/day for 5 days once every 4 weeks) [1] and extended-dosing regimen (75 mg/m²/day for 6 weeks once every 8 weeks) [2]. This response proportion is indistinguishable from that of dacarbazine.

We wished to test the use of TMZ in the neoadjuvant setting. First, given the smaller tumor burden, it was possible that these patients would be more responsive to treatment. Secondly, the advantage of a neoadjuvant therapy is that it provides in vivo assessment of tumor responsiveness to systemic therapy, potentially eradicates micrometastatic disease, and may improve the surgical result. Thirdly, it also could provide an opportunity to obtain tumor samples before and after treatment to study variates of response and effects of treatment on tumor characteristics.

We conducted a phase II neoadjuvant trial of TMZ in patients with potentially resectable stage III or IV melanoma. We collected fresh tumor before and after treatment to see if it is feasible to collect routinely cryopreserved tumor tissue that can be used for genetic analysis. We measured methylation of the O-6-methylguanine-DNA methyltransferase (MGMT) promoter in tumor samples since there is evidence that one mechanism of drug resistance to alkylating agents in general, and to TMZ in particular, is expression of MGMT, an enzyme that repairs guanine methylation caused by TMZ. We also planned to carry out DNA microarray analyses on tumor samples. The primary goal of the trial was to determine the objective response rate to TMZ in the neoadjuvant setting to see if the response rate in the neoadjuvant setting was higher than previously reported for patients with widely metastatic melanoma. A secondary goal was to determine whether the neoadjuvant setting would allow us to obtain cryopreserved...
pretreatment and post-treatment tumor samples of sufficient quality for genetic analysis.

materials and methods

patients

Patients were eligible if they had palpable American Joint Committee on Cancer version 2001 stage III (N1b, N2b, N2c, or N3) or stage IV (M1a) cutaneous melanoma and were potential candidates for complete surgical resection. Patients had to be at least 18 years old with a Karnofsky performance status ≥60% and have adequate organ function defined as follows: absolute neutrophil count ≥1500/µl, platelets ≥100 000/µl, creatinine level ≤2 mg/ml, and transaminases, bilirubin, alkaline phosphatase <1.5× upper limit of normal. Patients were excluded if they had received prior chemotherapy (prior immunotherapy was allowed), a history of medical illnesses that were poorly controlled or whose control might be jeopardized by the complications of therapy. Patients were not allowed to be on other melanoma therapy or use high-dose vitamins or herbs.

This clinical trial was approved by the Memorial Hospital Institutional Review Board and all patients signed written informed consent before participating.

treatment

For this trial, we used the extended-dosing regimen of TMZ consisting of 8-week cycles of TMZ 75 mg/m2/day by mouth for 6 weeks followed by 2 weeks off. We chose the extended-dosing schedule over the standard 5-day schedule because the extended-dosing schedule results in 50% more drug exposure and our prior experience indicated that the extended-dosing schedule was better tolerated. Also, we wished to compare directly the results in the neoadjuvant setting with our prior results in patients with unresectable disease [2]. Before starting treatment, patients underwent computed tomography (CT) scan of chest/abdomen/pelvis and PET scan within 3 weeks of starting therapy. Within 2 weeks of starting therapy, patients had a complete blood count (CBC) with differential, comprehensive chemistry screening, lactate dehydrogenase (LDH), T-cell subset analysis (CD3+, CD4+, and CD8+), prothrombin/partial thromboplastin time (PT/PTT), and electrocardiogram. Premenopausal women had a pregnancy test. Patients also had a pretreatment tumor biopsy carried out either as an excisional biopsy, Tru-Cut biopsy, or a punch biopsy. Tumor was trimmed of non-tumor tissue if necessary, flash frozen, and stored at −80°C until needed.

Patients were seen at week 4 for a physical examination and a CBC. If there was no evidence of disease progression, the patient completed the final 2 weeks of TMZ therapy. During weeks 7–8, the patients underwent a repeat CT scan of the chest/abdomen/pelvis. Patients were seen again at the end of each cycle (week 8) for a physical examination, CBC with differential, comprehensive chemistry screening, LDH, and T-cell subset analysis (CD3+, CD4+, and CD8+).

If there was evidence of tumor shrinkage or stable disease (SD) at the end of the first cycle, patients continued on to cycle 2 and continued therapy as long as tumor shrinkage was evident. Upon completion of TMZ therapy, the patients underwent surgical resection. Any patient with SD for two cycles came off TMZ treatment and underwent surgical resection. If at any time, there was evidence of progression of disease, including at the week-4 physical examination, TMZ was discontinued and the patient went to surgery. At surgery, a portion of the resected tumor was flash frozen and stored at −80°C until needed.

response criteria

Tumor responses were evaluated using RECIST criteria (version 1.0). Initially, patients were considered assessable for response if they had completed a full cycle of TMZ and had undergone a week-8 tumor evaluation. During the study, this was changed to a more conservative standard in which patients who were found to have progressed at the first visit (week 4 of cycle 1) would also be considered assessable for response.

oligonucleotide arrays

Tumor specimens for oligonucleotide array analysis were trimmed of nonmelanoma tissue, placed in a freezing vial, flash frozen in liquid nitrogen, and stored at −80°C. RNA was extracted from cryopreserved specimens using a RNeasy kit (QIAGEN, Valencia, CA). Before labeling, 25–50 ng of total RNA was run on a RNA 6000 Nano Assay (Agilent, Santa Clara, CA) to assess quality of the RNA. After reverse transcription with an oligo(dT)-T7, double-stranded complementary DNA was generated, linearized, amplified, and labeled with biotinylated nucleotides. The labeled material was hybridized on to an Affymetrix U133 plus 2.0 array (~45 000 transcripts) for 16 h at 45°C. Posthybridization staining, washing, and scanning were processed according to manufacturer’s recommendations.

promoter methylation of MGMT gene in pretreatment melanoma tumor samples

MGMT promoter methylation studies were carried out by OncoMethylome Sciences, Inc. (Durham, NC) on paraffin-embedded tumor specimens using methylation-specific PCR, as described previously [3]. Briefly, 40 μg of the tumor sample was deparaffinized, digested, and the genomic DNA was isolated by a standard phenol–chloroform method. Up to 1.5 μg of DNA was treated by sodium bisulfite resulting in the deamination of nonmethylated cytosine bases to uracil bases. Quantitative PCR for the methylated version of the MGMT gene and for the β-actin gene was then carried out. Samples with <1000 copies of β-actin were considered nonevaluable. The normalized ratio of methylated MGMT copy number to β-actin copy number was then calculated. Samples with methylated MGMT : β-actin ratio of ≥12 were considered methylated, between 5 and 12 considered intermediate methylation status, and a ratio of ≤5 was considered nonmethylated.

biostatistics

A Simon two-stage design was used whereby a 20% response rate was considered not promising, a 40% response rate was considered promising, and the probabilities of a type I error (falsely accepting a non-promising therapy) and type II error (falsely rejecting a promising therapy) were set at 0.10 and 0.10, respectively. In the first stage of this design, 17 assessable patients were accrued. If at least 4 patients achieved a response among these 17 patients, then an additional 20 patients would be accrued to the second stage. If three or fewer responses were seen, the study would be terminated and declared negative. Due to a change in the definition of an assessable patient, 19 patients were assessable. At the end of the trial, 11 responses had been observed of 37, then TMZ would have been considered worthy of further investigation. This design yielded at least a 0.90 probability of a positive result if the true response rate was at least 40% and yielded a 0.90 probability of a negative result if the true response rate was 20%.

results

patient characteristics

From October 2005 to February 2008, 27 patients signed informed consent to participate in this study. Five patients were not enrolled because either their pretreatment evaluation revealed metastatic disease (three patients) or their pretreatment tumor biopsy did not contain tumor (two patients). Twenty-two patients were enrolled and are assessable...
for toxicity. Of these, three patients are not assessable for response because either they withdrew consent before the week-4 evaluation (two patients) or were found to be ineligible for the study because they had a mucosal primary (one patient). Overall, 19 patients were assessable for response.

The patient characteristics are shown in Table 1. Of the 19 patients assessable for response, 14 had stage III and 5 had stage IV M1a melanoma. Most patients (12 of 19) received a single cycle or less of TMZ and were taken off treatment due to progression of disease. Six patients received two cycles; one patient, who had a complete response (CR), received three cycles of TMZ.

**clinical responses**

There were two clinical CRs and one clinical partial response (PR; overall response rate 16%); all responders had stage III disease. One complete responder underwent completion lymph node dissection, which confirmed the absence of tumor. That patient remains free of disease 14 months later. The other complete responder did not undergo completion lymph node dissection and is clinically free of disease 15 months after completing three cycles of TMZ. The partial responder underwent a completion lymph node dissection, which revealed melanoma in 2 of 16 lymph nodes with extensive necrosis. That patient remains free of disease 27 months after surgery.

Four patients had SD, underwent complete surgical excision of their melanoma, and remain free of disease. Twelve patients had progression of disease, which was detected at week 4 or 8 in 11 of the patients. One patient with a mixed response received 4 weeks of cycle 2 before progression of disease was evident. Of these 12 patients, 6 were rendered free of disease by surgery although 5 ultimately recurred. One patient remains free of disease 6 months after surgery. Five patients were felt to be ineligible for resection either because of locally advanced disease (four patients) or due to new metastatic disease (one patient); another underwent resection of locally advanced disease but could not be resected completely. All were treated with systemic therapy and three of these patients have gone on to respond to immunotherapy and remain alive with minimal disease 13–16 months after progressing on TMZ. Another patient is alive with disease 7 months after progressing on TMZ, and two patients died 7 and 9 months after progressing on TMZ.

**tumor MGMT promoter methylation**

Adequate pretreatment tumor specimens were available from four patients for analysis of MGMT promoter methylation; one patient also had post-treatment tumor available. In another five patients, only post-treatment tumor was available for analysis (Table 2). For the remaining patients, there was not adequate amount of tumor material to detect >1000 copies of β-actin and so were considered nonassessable. In all evaluable specimens, the MGMT promoter was found to be unmethylated indicating that the MGMT gene was not silenced. Although only a subset of patients could be analyzed, two of the patients analyzed were the complete responders. These data indicate that in melanoma, epigenetic silencing of MGMT is not required for response to TMZ. However, we cannot rule out the possibility that the tumors of the responding patients had low levels of MGMT despite the lack of promoter methylation.

**tumor microarray analysis**

Adequate pretreatment tumor material could be obtained from 16 patients (3 responders, 13 nonresponders). In three patients, biopsies were thought not to include sufficient amounts of tumor material. For seven of the nonresponding patients, post-treatment tumor was also obtained. For one nonresponding patient, only post-treatment tumor was available. Because there was no post-treatment tumor from the two complete responders, we could not obtain post-treatment tumors from these patients.

We were able to carry out microarray analysis on all 16 pretreatment samples, which indicates that in 84% of patients, we could obtain adequate pretreatment tumor specimens for genetic analysis.

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Total number treated</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assessable for tumor response</td>
<td>19</td>
</tr>
<tr>
<td>Gender</td>
<td>6 women, 13 men</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>59 (41–79)</td>
</tr>
<tr>
<td>Stage III</td>
<td>14</td>
</tr>
<tr>
<td>N1b</td>
<td>5</td>
</tr>
<tr>
<td>N2b</td>
<td>4</td>
</tr>
<tr>
<td>N2c</td>
<td>3</td>
</tr>
<tr>
<td>N3</td>
<td>2</td>
</tr>
<tr>
<td>Stage IV, M1a</td>
<td>5</td>
</tr>
<tr>
<td>Cycles of TMZ &lt;1</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

*Two patients withdrew consent before week-4 evaluation. One patient not eligible due to mucosal primary. TMZ, temozolomide.

**Table 2. Analysis of tumor MGMT promoter methylation**

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Best clinical response</th>
<th>Pretreatment tumor</th>
<th>Post-treatment tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>CR</td>
<td>Unmethylated</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>CR</td>
<td>Unmethylated</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>POD</td>
<td>Unmethylated</td>
<td>Unmethylated</td>
</tr>
<tr>
<td>5</td>
<td>POD</td>
<td>Unmethylated</td>
<td>Unmethylated</td>
</tr>
<tr>
<td>6</td>
<td>POD</td>
<td>Unmethylated</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>POD</td>
<td>Unmethylated</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>POD</td>
<td>Unmethylated</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>POD</td>
<td>Unmethylated</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>POD</td>
<td>Unmethylated</td>
<td></td>
</tr>
</tbody>
</table>

MGMT, O-6-methylguanine-DNA methyltransferase; CR, complete response; POD, progression of disease.
toxicity

As expected, neoadjuvant TMZ was very well tolerated with few toxic effects grade II or more (Table 3). Three patients experienced grade III lymphopenia although no opportunistic infections were observed. Three patients had grade III elevations of transaminases, which was self-limited.

discussion

Previous experience with neoadjuvant therapy in melanoma is limited. Buzaid et al. [4] reported the use of neoadjuvant biochemotherapy and saw an objective response rate of 48% with 10% CRs. This was similar to the response rate observed in patients with metastatic melanoma [5–7]. Interestingly, 2 of 5 patients who were found to have pathological CRs at surgery had experienced less than a CR clinically (1 PR, 1 SD). Moschos et al. [8] reported that 1 month of neoadjuvant high-dose interferon-α2b induced clinical objective responses in 55% of patients, which is substantially higher than the response in stage IV melanoma patients [9–12]. As with neoadjuvant biochemotherapy, the clinical assessments of antitumor effects underestimated the CRs documented at surgery. Both patients who were found to have had a pathological CR had experienced only a clinical PR.

In our trial, we used extended-dosing TMZ in the neoadjuvant setting. We had previously observed a 12.5% response proportion in unresectable stage IV patients [2] and we wanted to determine whether the response proportion would be higher in the neoadjuvant setting. We observed 3 responses in the first 19 patients, 2 CRs and 1 PR, for a response proportion of 16%. In contrast to the neoadjuvant trials with biochemotherapy or interferon-α2b, there were no pathological CRs among the patients with residual palpable disease at the time of surgery. We were not able to reject the null hypothesis that the true response proportion is ≤20% and so ceased accrual. This response proportion is consistent with our phase II data in metastatic patients [2] and indicates that patients treated in the neoadjuvant setting are not more likely to respond than patients with stage IV, although in our previous experience with 45 stage IV melanoma patients, we saw no CRs.

Since these neoadjuvant patients all had palpable tumor before treatment and later underwent surgical resection, there was an opportunity to obtain tumor tissue before and after treatment. A secondary objective of our study was to test the feasibility of obtaining adequate cryopreserved tumor before and after treatment for genetic analysis.

We were able to analyze pretreatment tumor from 16 of 19 (84%) patients by microarray analysis; in 3 patients, pretreatment core biopsies were thought not to contain identifiable tumor material.

We tested pretreatment tumor samples for MGMT promoter methylation. In glioblastoma multiforme, response to TMZ treatment is correlated with epigenetic silencing of MGMT by promoter methylation [13, 14]. However, past reports by us [2] and others [15, 16] have failed to find a correlation in melanoma between response to TMZ and methylation of the MGMT promoter. In this study, we were able to obtain adequate tissue for MGMT promoter methylation in pretreatment tumors only in four patients. This was due partially to the fact that in many patients, only a small core of tissue could be obtained and priority was given to the microarray analyses. Fortunately, two of these patients experienced CRs on treatment allowing us to conclude that MGMT promoter hypomethylation is not required for response to TMZ in melanoma.

We conclude that in the neoadjuvant setting, TMZ can induce CRs but that overall, the chance of clinical response is similar to that in the metastatic setting. We could not obtain adequate pretreatment cryopreserved tumor samples in every patient in the neoadjuvant setting. The major limitation was that core biopsies of pretreatment lymph nodes sometimes did not contain tumor cells. Obtaining post-treatment tumor material was more reliable, but the most informative patients—those who had undergone a CR—clearly did not have post-treatment tumor available. Future studies should take advantage of new platforms, such as the Illumina cDNA-mediated annealing selection, extension, and ligation (DASL) assay, that permit the use of paraffin-embedded material to ensure that there is adequate tumor.

funding

Schering-Plough Corporation.

acknowledgements

We thank Jacqueline Simproonio for data management, Ruth-Ann Roman and Virginia Murphy for expert nursing care, and Greg Jones at OncoMethylome Sciences, Inc. for carrying out the MGMT promoter methylation assays. This study is presented, in part, at the 2008 Annual Meeting of the Society for Clinical Oncology, Chicago, IL.

references


