Cancer Therapy: Preclinical

Humanized Bone Marrow Mouse Model as a Preclinical Tool to Assess Therapy-Mediated Hematotoxicity

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Abstract

Purpose: Preclinical in vivo studies can help guide the selection of agents and regimens for clinical testing. However, one of the challenges in screening anticancer therapies is the assessment of off-target human toxicity. There is a need for in vivo models that can simulate efficacy and toxicities of promising therapeutic regimens. For example, hematopoietic cells of human origin are particularly sensitive to a variety of chemotherapeutic regimens, but in vivo models to assess potential toxicities have not been developed. In this study, a xenograft model containing humanized bone marrow is utilized as an in vivo assay to monitor hematotoxicity.

Experimental Design: A proof-of-concept, temozolomide-based regimen was developed that inhibits tumor xenograft growth. This regimen was selected for testing because it has been previously shown to cause myelosuppression in mice and humans. The dose-intensive regimen was administered to NOD.Cg-PrkdcscidIL2rgtm1Wjl/Sz (NOD/SCID/γc null), reconstituted with human hematopoietic cells, and the impact of treatment on human hematopoiesis was evaluated.

Results: The dose-intensive regimen resulted in significant decreases in growth of human glioblastoma xenografts. When this regimen was administered to mice containing humanized bone marrow, flow cytometric analyses indicated that the human bone marrow cells were significantly more sensitive to treatment than the murine bone marrow cells and that the regimen was highly toxic to human-derived hematopoietic cells of all lineages (progenitor, lymphoid, and myeloid).

Conclusions: The humanized bone marrow xenograft model described has the potential to be used as a platform for monitoring the impact of anticancer therapies on human hematopoiesis and could lead to subsequent refinement of therapies prior to clinical evaluation.

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Introduction

Murine xenograft models are one of the primary tools used for screening new therapeutic compounds and regimens (1). One major limitation of using murine xenograft studies to determine therapeutic efficacy, however, is that significant interspecies differences in drug sensitivity can exist between mouse- and human-derived cells. It is possible that the levels of a therapeutic compound reached in a mouse xenograft model may not be achievable in humans due to differential profiles of ADME (absorption, distribution, metabolism, and excretion) and toxicity. Therefore, although a regimen may exhibit an acceptable toxicity and efficacy profile in mice, this may not necessarily be the case in patients.

Furthermore, in vitro clonogenic assays have demonstrated that human and mouse hematopoietic cells show a wide diversity in sensitivity to many anticancer drugs; mouse hematopoietic cells in many cases exhibit increased resistance to compound exposure compared with human hematopoietic cells (2–5). Masubuchi and colleagues have previously shown differential sensitivities of mice, dog, and human bone marrow cells exposed to camptothecin derivatives. For example, camptothecin-like compounds, SN-38 and topotecan, exhibited differential interspecies resistance, with mouse colony forming unit (CFU) being the most resistant to treatment (4). However, interspecies differences in sensitivity were not always observed. Three other compounds (DX-8951F, 9-aminocamptothecin, and
We first developed a dosing regimen consisting of O6-benzylguanine and temozolomide (O6-BG/TMZ) on hematopoiesis (6). As a proof of concept, we chose to determine the impact of this regimen on human hematopoietic cells with a regimen related to clinical practice and strain is to treat mice reconstituted with human hemato-


camptothecin) showed fairly similar sensitivities among all 3 species. Studies by Kurtzberg and colleagues (3) indicated significant differences in sensitivities for tubulin-binding agents; the IC90 values for vincristine and paclitaxel were 30 and 27 nmol/L and 3 and 9 nmol/L for mouse CFU-GM (granulocyte macrophage) and human CFU-GM, respectively. In addition, IC90 values for taxidotin treatment were more than 300 nmol/L for mouse CFU-GM and 65 nmol/L for human CFU-GM.

Evaluation of human hematopoietic toxicity in immunodeficient mice could represent an additional benchmark in the final screening and selection of new therapeutics. This in vivo screening approach would take into account the influence of the bone marrow microenvironment on damaged cells or the cycling kinetics of hematopoietic cells following treatment. An unexplored use of the NOD.Cg-PrkdcscidIL2rgtm1Wjl mouse strain is to treat mice reconstituted with human hematopoietic cells with a regimen related to clinical practice and determine the impact of in vivo treatment on human hematopoiesis (6). As a proof of concept, we chose to evaluate the impact of a combination therapy consisting of O6-benzylguanine and temozolomide (O6-BG/TMZ) on human hematopoiesis in vivo because it is currently being evaluated in clinical trials and the main dose-limiting toxicity in these patients is myelosuppression (7–10). We first developed a dosing regimen consisting of O6-BG/TMZ, followed by stem cell rescue that would significantly inhibit the growth of TMZ-resistant human xenografts in NOD.Cg-Prkdcscid IL2rgtm1Wjl mice. The underlying strategy of this regimen is to prevent repair of TMZ-mediated DNA damage by inhibiting the DNA repair protein, O6-methylguanine-DNA methyltransferase (MGMT; refs. 11–14). Previous studies have shown that numerous cancers can express high levels of MGMT, and therefore can efficiently repair TMZ-mediated DNA damage, decreasing the efficacy of tumor cell kill (15). In addition, a variety of tumors can be sensitized to alkylating agents both in vitro and in xenograft studies with the addition of O6-BG, a direct inhibitor of MGMT activity (11, 13, 14, 16). The downside of this approach is that both immature and mature hematopoietic cells can be extremely sensitive to this regimen due to low levels of endogenous DNA repair activity (17).

In this study, the delivery of 2 cycles of a high-dose regimen of O6-BG/TMZ in combination with stem cell rescue significantly inhibited the growth of a TMZ-resistant glioma. This course of therapy was then used to test the hypothesis that administration of the regimen would be toxic to human hematopoietic cells in vivo. NOD/SCID/γ-chainnull mice were transplanted with human CD34+ cells and reconstitution was confirmed 1 month posttransplantation by monitoring the peripheral blood (PB) for human cell chimerism in the transplanted mice. Treatment with O6-BG/TMZ resulted in significant loss of human-derived hematopoietic cells in the bone marrow and enumeration of the total number of human and mouse cells in vehicle versus treated mice indicated that the human bone marrow cells were significantly more sensitive to treatment compared with the murine-derived bone marrow cells. This proof-of-concept study indicates that the use of NOD/SCID/γ-chainnull mice with humanized bone marrow can be used as an in vivo toxicity measure of human hematopoiesis following drug treatment and holds merit as a model for improving screening strategies used to develop and test novel compounds and dosing regimens.

Materials and Methods

Isolation of umbilical cord blood CD34+ cells

All protocols were approved by Indiana University School of Medicine’s Institutional Review Board (IRB) and St. Vincent Hospital’s IRB (Indianapolis, IN). Samples of umbilical cord blood (UCB) were collected from normal, full-term infants delivered by cesarean section, and the CD34+ cells were isolated using the CD34 MicroBead Kit and VarioMACS Separator (Miltenyi Biotech Inc) according to the manufacturer’s instructions. Following magnetic bead separation, the viability of the isolated CD34+ cells was routinely 95%. The quality of the isolated CD34+ cells was analyzed by a CFU assay as described in the following text on an aliquot of the CD34+ cells used for transplantation. The frequency of clonogenic cells in UCB products used for transplant in experiments I and II was similar, with 60 to 65 colonies per 2 × 105 CD34+ cells plated.

Clonogenic survival assays

CFU assays were conducted using enriched human CD34+ cells (MethoCult GF H4434; Stem Cell Technologies, Inc.) and unfractionated murine bone marrow (MethoCult GF M3434), or bone marrow from NOD/SCID/γ-chainnull transplanted with human CD34+ cells.
The cells were seeded in triplicate dishes at concentrations of $2 \times 10^5$ for human CD34+ cells and $2 \times 10^5$ for murine bone marrow to obtain 60 to 70 colonies per 35-mm dish. For survival assays, cells were exposed to drug combinations (O6-BG, TMZ, or O6-BG/TMZ) before plating. For analysis of human cells from the bone marrow of NOD/SCID/γc null transplants, chimeric human–mouse bone marrow was plated at $2 \times 10^5$ bone marrow cells per dish. After 10 to 14 days of incubation at 37°C in 5% CO2, CFU-GM and burst-forming units–erythroid (BFU-E) were enumerated using an Axiovert 25 inverted light microscope. For assays using clonogenic SF767 cells, cells were seeded at 150 cells/well in triplicate in 6-well plates and allowed to grow overnight in Iscove’s modified Dulbecco’s medium (IMDM) with supplements (10% cosmic calf serum, 1% pen/strep, and 1% glutamine.) The cells were treated the next day and allowed to grow for 13 days at 37°C. Following incubation, the cells were rinsed with PBS, fixed with 0.05% methylene blue, and counted visually.

Animals
A breeding colony of NOD/SCID/γc null mice (6) was established at the Laboratory Animal Research Center and maintained by the In Vivo Therapeutics Core at the Indiana University Simon Cancer Center (IUSCC, Indianapolis, IN). BALB/c and C57BL/6J inbred mice were purchased from Harlan Laboratories Inc. All protocols and establishment of predeath endpoints—decreases in body weight, activity, and grooming—were approved by the Institutional Animal Care and Use Committee.

Xenograft study
The human glioblastoma cell line SF767 was originally developed by The Brain Tumor Research Center, University of California at San Francisco, and has been previously described (18). The human glioblastoma cell line SF767 was tested for drug sensitivity of the cell populations associated with this treatment in the clinic (8, 9). To gain insight into the drug sensitivity of the cell populations to be investigated (i.e., primary murine hematopoietic cells, primary human hematopoietic cells, and glioma cells), clonogenic survival assays were set up in the absence and presence of 20 μM/L O6-BG and increasing doses of TMZ (0–200 μM/L; Fig. 1). Surrogate bone marrow containing 0.2% endotoxin-free bovine serum albumin and injected into the lateral tail vein of each animal.

Chemotherapy administration
O6-BG (Sigma-Aldrich) was dissolved in 40% polyethylene glycol-400 (v/v) and 60% saline (v/v). TMZ (LKT laboratories, Inc.) was sonicated in PBS. The myeloablative dosing regimen consisted of 30 mg/kg O6-BG, followed by 80 mg/kg TMZ 1 hour later and 15 mg/kg O6-BG 7 hours later (O6-BG/TMZ). One treatment cycle consisted of 3 consecutive days of treatment with this dosing regimen. Two days after a treatment cycle, $8 \times 10^6$ fresh murine bone marrow cells were injected intravenously.

Analysis of human cell engraftment
Mice were sacrificed at 6 and 11 days postinjection, and the bone marrow and spleen were prepared as previously described (19). Human cell engraftment was measured by human CD45 immunostaining. The proportion of engraftment in various lineages was determined by immunostaining and flow cytometric analysis as previously described using a Becton Dickinson FACS Calibur and CellQuest software (19). The lack of cross-reactivity of human-specific antibodies with murine cells was confirmed in each experiment by staining bone marrow from a nontransplanted mouse with each antibody combination. Cells were stained with allophycocyanin (APC)-conjugated anti-human CD45 (anti–HLA-1; Becton Dickinson Immunocytometry) alone or in combination with phycoerythrin (PE)-conjugated anti-human CD33 (anti–Leu-M9; Becton Dickinson). Identical aliquots were stained with APC-conjugated anti-human CD34 (clone 581; Pharmingen) in combination with anti-human CD19-PE (Pharmingen).

Statistical analyses
Generalized linear mixed model with mouse as the random effect was used to compare tumor volume between O6-BG/TMZ and vehicle treatments. Two-tailed $t$ tests were conducted to determine significance of vehicle treatment versus drug treatment for tumor weights, clonogenic survival assays, and flow cytometric analyses.

Results
Development of a proof-of-concept TMZ-based regimen that results in a significant decrease in tumor xenograft growth
A combination treatment consisting of O6-BG and TMZ was selected to serve as the proof-of-concept treatment for development of the in vivo human toxicity model because myelosuppression is the major dose-limiting toxicity associated with this treatment in the clinic (8, 9). To gain insight into the drug sensitivity of the cell populations to be investigated (i.e., primary murine hematopoietic cells, primary human hematopoietic cells, and glioma cells), clonogenic survival assays were set up in the absence and presence of 20 μM/L O6-BG and increasing doses of TMZ (0–200 μM/L; Fig. 1). Surrogate bone marrow...
Assume that the sensitivity of the murine bone marrow for the drug combination studied here, it was reasonable to presence and absence of O6-BG (Fig. 1A). Therefore, at least versus tumor for O6-BG/TMZ at all TMZ concentrations, human versus presence of O6-BG. At days 10 to 12, hematopoietic colonies (human and tumor cells were exposed to increasing doses of TMZ in the absence or presence of O6-BG/TMZ. A, BALB/c, C57BL/6N, and NOD/SCID/γ-chainnull mice bone marrow cells were exposed to increasing doses of TMZ in the absence or presence of O6-BG. At days 10 to 12, hematopoietic colonies (human and mouse) were enumerated. SF767 colonies were scored visually by counting methylene blue-stained colonies. P < 0.0001 for TMZ versus O6-BG/TMZ for all cell types, mouse versus human for O6-BG/TMZ, mouse versus tumor for O6-BG/TMZ at all TMZ concentrations, human versus tumor for O6-BG/TMZ at all TMZ concentrations except 200 μmol/L TMZ. HC, hematopoietic cells; NSG, NOD/SCID/γ-chainnull bone marrow cells.

toxicity studies of new compounds and regimens are commonly evaluated in immunocompetent mice. Because immunodeficient mice would be required for engraftment of human CD34+ cells, it was important to compare the drug sensitivity of murine clonogenic cells derived from immunocompetent (BALB/c, C57BL/6N) and immunodeficient mice (NOD/SCID/γ-chainnull). Murine bone marrow cells were set up in the absence or presence of drug combinations, and sensitivity of clonogenic cells was determined by CFU assays. The dose-dependent sensitivities of clonogenic cells derived from BALB/c, C57BL/6N, and NOD/SCID/γ-chainnull mice were similar to O6-BG on MGMT-mediated DNA repair. We next determined to what extent the in vitro sensitivities of the cell populations correlated with in vitro anti-tumor efficacy and toxicity to the bone marrow compartment in NOD/SCID/γ-chainnull mice. To accomplish this, it was essential to develop an efficacious O6-BG/TMZ regimen that resulted in significant tumor kill in vivo. Treatment cycles containing increasing doses of TMZ were first tested for toxicity in NOD/SCID/γ-chainnull mice (Fig. 2A and B). Each cycle consisted of 3 consecutive days of treatment with 30 mg/kg O6-BG, followed 1 hour later by 40, 60, or 80 mg/kg TMZ, and a second bolus of O6-BG delivered 7 hours later. The O6-BG double bolus has been shown previously to deplete MGMT for at least 18 hours and prevent repair of TMZ-mediated DNA damage (21). Survival was determined using a predeath endpoint system (see Material and Methods). Two cycles of treatment initiated a week apart resulted in morbidity in more than 85% of the NOD/SCID/γ-chainnull mice (Fig. 2A). Histopathologic evaluation of major organs (brain, colon, kidney, liver, lung, heart, spleen, and stomach) was done on 2 mice treated with vehicle and 4 mice treated with O6-BG and followed by TMZ at 80 mg/kg; no gross abnormalities were noted. However, in mice treated with O6-BG and TMZ at 80 mg/kg, bone marrow cellularity was decreased by at least 70% in the O6-BG/TMZ-treated mice compared with vehicle-treated mice when analyzed at the predeath endpoint (data not shown). In contrast, when an autologous murine bone marrow transplant was given 2 days after each cycle of drug administration, all mice maintained normal body weight and morbidity was prevented (Fig. 2A). We next tested the efficacy of this regimen using NOD/SCID/γ-chainnull mice containing ectopic SF767 glioblastoma xenografts (Fig. 2B). In 2 independent experiments, mice received 2 cycles of either vehicle control or O6-BG/TMZ.

Figure 1. Determination of in vitro sensitivities to TMZ and combination O6-BG/TMZ. A, BALB/c, C57BL/6N, and NOD/SCID/γ-chainnull bone marrow cells were exposed to increasing doses of TMZ in the absence or presence of O6-BG. At days 10 to 12, hematopoietic colonies (human and mouse) were scored visually by inverted light microscopy. B, human CD34+ cells, NOD/SCID/γ-chainnull mice bone marrow cells, and SF767 tumor cells were exposed to increasing doses of TMZ in the absence or presence of O6-BG. At days 10 to 12, hematopoietic colonies (human and mouse) were enumerated. SF767 colonies were scored visually by counting methylene blue-stained colonies. P < 0.0001 for TMZ versus O6-BG/TMZ for all cell types, mouse versus human for O6-BG/TMZ, mouse versus tumor for O6-BG/TMZ at all TMZ concentrations, human versus tumor for O6-BG/TMZ at all TMZ concentrations except 200 μmol/L TMZ. HC, hematopoietic cells; NSG, NOD/SCID/γ-chainnull bone marrow cells.
treatment. After each 3-day treatment cycle, $8 \times 10^6$ autologous murine bone marrow cells were transplanted via tail vein and tumor growth was monitored over time. Following 2 cycles of treatment and stem cell rescue, a significant delay in tumor progression was observed in vehicle- versus O6-BG/TMZ-treated mice (Fig. 2C). At 8 weeks posttreatment, mice were sacrificed for tumor and bone marrow analysis. Tumors from drug-treated mice weighed significantly less than tumors from vehicle-treated mice (Fig. 2D). Bone marrow cellularity of vehicle-treated mice versus mice receiving O6-BG/TMZ and stem cell rescue was not significantly different (data not shown). These data indicate that significant decreases in growth of alkylator-resistant tumor cells can be obtained with high-dose O6-BG/TMZ when combined with autologous stem cell rescue and provided us with a relevant proof-of-concept regimen that could be used to monitor impact of the treatment on human hematopoiesis in vivo.

In vivo biotoxicity assay to evaluate impact of anticancer therapies on normal human hematopoietic cell function

Our next objective was to determine to what extent the high-dose O6-BG/TMZ regimen that effectively kills tumor cells might affect human hematopoiesis in vivo. NOD/SCID/γcnull mice were sublethally irradiated and transplanted with $1 \times 10^6$ human CD34+ cells (Fig. 3A). At 4 weeks posttransplant, a sample of PB was analyzed for the
presence of human CD45<sup>+</sup> cells to confirm human cell engraftment (Fig. 3A and Table 1). Next, cohorts of mice were treated with vehicle or 1 cycle of O6-BG/TMZ. Bone marrow was harvested at 6 or 11 days posttreatment and analyzed for the percentage and total number of human versus mouse cells (Table 1). Our objective was to determine the maximum impact of the treatment regimen on human hematopoiesis. Therefore, we analyzed the bone marrow at 6 days posttreatment because we have previously found that the nadir in human myeloid progenitor numbers is typically reached at day 6 following TMZ treatment in vitro (data not shown). In addition, the bone marrow was also analyzed at 11 days posttreatment in one experiment to assess marrow function and potential recovery after the nadir period. For both experiments I and II, the fold decrease in the number of human CD45<sup>+</sup> cells in vehicle versus O6-BG/TMZ-treated mice was statistically significant.

Figure 3. Human toxicity model to assess impact of treatment on human bone marrow cells. A, human CD34<sup>+</sup> cells were isolated from UCB and transplanted into sublethally irradiated NOD/SCID/γ-chain<sup>-/-</sup> mice. At 4 weeks posttransplant, a sample of PB was analyzed from each mouse to confirm that all mice were similarly engrafted. Cohorts of mice received vehicle or 1 cycle of the O6-BG/TMZ regimen. At 6 to 11 days posttreatment, the bone marrow was harvested and analyzed via flow cytometry for the presence of human cells. B, at 6 days posttreatment, the bone marrow was harvested, counted, and analyzed via flow cytometry to determine the number of human CD34<sup>+</sup> cells in the bone marrow from vehicle- and O6-BG/TMZ-treated mice. *, P < 0.001 for vehicle- versus drug-treated mice. C, the bone marrow from vehicle- and O6-BG/TMZ-treated mice was analyzed for the presence of human CFU cells and analyzed 12 days after plating. *, P < 0.001 for vehicle- versus drug-treated mice.
significant (Table 1, \(P < 0.001\)). Although different baseline levels of human engraftment in the bone marrow were observed in experiments I and II (see vehicle-treated mice, Table 1), \(2 \times 10^5\) CD34\(^+\) cells were transplanted per animal in both experiments. It has been our experience that the frequency of SCID-repopulating cells can vary widely among different cord blood products. In either situation (high or low human cell engraftment), the drug sensitivity of the human bone marrow cells was similar. In experiment I, a 9-fold decrease in the number of human cells in the bone marrow was observed following O\(^6\)-BG/TMZ treatment. In contrast, only a 1.5-fold decrease in the number of murine bone marrow cells was observed in these same mice (Table 1). In addition, because of high levels of human engraftment in experiment I, the spleen was also analyzed for human hematopoietic cells; significant decreases in human cells was observed in O\(^6\)-BG/TMZ-treated mice compared with vehicle-treated mice (data not shown). In experiment II, total human engraftment pretreatment was lower than in experiment I, which is not unexpected, because different cord blood products were used in each experiment (Table 1; see % huCD45\(^+\) cells in the PB pretreatment). This also enabled us to see whether lower levels of human chimerism would result in a different toxicity profile (human vs. mouse) from that observed in experiment I. Differential sensitivity of human and mouse bone marrow cells was observed at 6 and 11 days postdrug treatment, with the human cells still exhibiting a higher sensitivity to the drug treatment than the mouse cells at both time points. A 13-fold decrease in human cell chimerism and a 1.5-fold decrease in mouse cell chimerism were observed at 6 days post–O\(^6\)-BG/TMZ treatment. At day 11 posttreatment, both a 3-fold decrease and a 1.2-fold decrease were observed for human and mouse cell chimerism, respectively (Table 1). In experiment II, it is important to note that the level of human cells in the PB pretreatment differed from the 2 cohorts used for the day 11 analysis (Table 1, see experiment II—% huCD45\(^+\) cells in the PB pretreatment at day 11). Human cells were detected at higher levels in the mouse cohort selected for drug treatment versus the cohort selected for vehicle treatment. Even with higher percentage of human cells in the PB of the cohort selected for O\(^6\)-BG/TMZ treatment, the fold decrease in human versus mouse bone marrow cells was still evident and statistically significant. These data collectively show that the human hematopoietic cells, even at different levels of overall human chimerism in the bone marrow, were significantly more sensitive to O\(^6\)-BG/TMZ treatment in vivo than mouse bone marrow cells.

The impact of treatment on multilineage differentiation typically found in the bone marrow of mice reconstituted with human cells was also evaluated (Figs. 3–5). The number of CD34\(^+\) cells significantly decreased following treatment (Fig. 3B). In contrast to vehicle-treated mice, CFU assays indicated that human erythroid and myeloid progenitor cells were no longer present in the bone marrow from drug-treated mice (Fig. 3C). The CD34\(^+\) cell population was further analyzed for different B-cell CD19\(^+\) subsets, typically found in humanized bone marrow (Fig. 4A and B). There was a significant decrease in the most primitive cells (CD34\(^+\), CD19\(^-\)), B-cell progenitors (CD34\(^+\), CD19\(^-\)), and more mature B-lymphoid cells (CD34\(^-\), CD19\(^+\)). In addition, a significant decrease in human CD33\(^+\) myeloid cells was evident (Fig. 5A and B).

### Table 1. Analysis of human versus mouse bone marrow cells posttreatment

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>% huCD45 in PB pretreatment (\text{day 0}^a)</th>
<th>Fold decrease after treatment (\text{day 11}^b)</th>
<th>% huCD45 in the bone marrow (\text{day 0}^c)</th>
<th>Total number of bone marrow cells (\times 10^6) (\text{day 0}^d)</th>
<th>Number of cells per femur (\times 10^6) (\text{day 11}^e)</th>
<th>Number of cells per femur (\times 10^6) (\text{day 6}^e)</th>
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<tr>
<td>I</td>
<td>Vehicle</td>
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<td></td>
<td></td>
<td>(18 \pm 2)</td>
<td>(3 \pm 1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O(^6)-BG/TMZ</td>
<td>(63 \pm 6.5)</td>
<td></td>
<td></td>
<td>(21 \pm 3)</td>
<td>(4 \pm 1)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Vehicle</td>
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<td></td>
<td></td>
<td>(13 \pm 3)</td>
<td>(6 \pm 3)</td>
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<tr>
<td></td>
<td>O(^6)-BG/TMZ</td>
<td>(11.5 \pm 5.6)</td>
<td></td>
<td></td>
<td>(1 \pm 1)</td>
<td>(4 \pm 1)</td>
<td>(13)</td>
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<tr>
<td></td>
<td>Vehicle</td>
<td>(5.2 \pm 0.8)</td>
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<td></td>
<td>(3 \pm 1)</td>
<td>(17 \pm 1)</td>
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<tr>
<td></td>
<td>O(^6)-BG/TMZ</td>
<td>(9.8 \pm 2.8)</td>
<td></td>
<td></td>
<td>(1 \pm 1)</td>
<td>(14 \pm 3)</td>
<td>(3)</td>
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\(\text{day 0}^a\): Mice were transplanted with human CD34\(^+\) cells and the PB analyzed at 1 month posttransplantation for the presence of human CD45\(^+\) cells. This analysis was done prior to O\(^6\)-BG/TMZ treatment.

\(\text{day 11}^b\): At 6 to 11 days post–O\(^6\)-BG/TMZ treatment, the bone marrow was harvested and the percentage of human cell chimerism (huCD45\(^+\)) was determined by flow cytometry.

\(\text{day 0}^c\): The total number of human and mouse bone marrow cells in vehicle- and O\(^6\)-BG/TMZ-treated mice was determined using a Coulter counter.

\(\text{day 0}^d\): Total number of human cells = total number bone marrow cells \(\times \%\) human cells. Total number of murine cells = total number bone marrow cells – total number human cells.

\(\text{day 0}^e\): The fold decrease between vehicle- and drug-treated human or mouse bone marrow cells = total number of human or mouse in vehicle-treated mice ÷ total number of human or mouse cells remaining in O\(^6\)-BG/TMZ-treated mice.
These studies indicate that differential sensitivities of mouse and human cells to cytotoxic dosing regimens are apparent both \textit{in vivo} and \textit{in vitro} and that the regimen sensitivities of human versus mouse may be even more pronounced in the bone marrow.

**Discussion**

Development of human xenograft models that focus on treatment efficacy and potential hematopoietic toxicities \textit{in vivo} could yield crucial insights into how to best translate basic research findings to the clinic. The main dose-limiting toxicity in more than 50% of anticancer drugs used in the past 15 years is therapy-mediated myelosuppression (22). Therefore, preclinical predictive models of this adverse event in humans could help guide the selection of new and safer therapeutic agents for clinical testing. Although humanizing the bone marrow compartment of NOD/SCID/\textit{γ} chain\textsuperscript{-}null mice cannot necessarily take into account all pharmacologic and interspecies differences in human and mouse, it can provide improved correlations of compound levels obtained \textit{in vivo} versus regimen efficacy and human hematotoxicity.

We built our study on the basis of the scientific questions observed from clinical observations in an effort to research improvements in therapy (e.g., from the bedside back to the bench). Middleton and colleagues originally determined the pharmacokinetic profile of TMZ in plasma from adult patients with a diagnosis of malignant melanoma. In patients who received TMZ starting at 200 mg/m\textsuperscript{2}/d, the range of peak TMZ levels (\(C_{\text{max}}\)) was 0.1 to 10.0 \(\mu\)g/mL or approximately 0.5 to 50 \(\mu\)mol/L in the plasma (23). Similar TMZ plasma levels were obtained in an adult glioma clinical study by Osterman and colleagues (24). In addition, when 267 mg/m\textsuperscript{2}/d was administered to pediatric brain tumor patients, levels of TMZ detected in the plasma in humans due to compound-induced myelotoxicity (2–5).

Figure 4. Analysis of human B-lymphoid cells in the bone marrow of NOD/SCID/\textit{γ} chain\textsuperscript{-}null mice following O\textdegree-BG/TMZ treatment. **A**, bone marrow from vehicle- and O\textdegree-BG/TMZ-treated mice was analyzed for the presence of CD34\textsuperscript{+} and CD19\textsuperscript{+} cells via flow cytometry. **B**, on the basis of flow cytometry and bone marrow cell numbers, the number of human CD34\textsuperscript{‘} CD19\textsuperscript{‘} (progenitors), CD34\textsuperscript{‘} CD19\textsuperscript{‘} (B-lymphoid progenitors), and CD34\textsuperscript{‘} CD19\textsuperscript{‘} (more mature B-lymphoid cells) were determined by flow cytometry. These data are representative examples of data from experiment I. Data are similar from experiment II, except that the level of overall human chimerism was lower in vehicle- and O\textdegree-BG/TMZ-treated mice. *, \(P < 0.0001\) for vehicle- versus drug-treated mice.

Figure 5. Analysis of human myeloid cells in the bone marrow of NOD/SCID/\textit{γ} chain\textsuperscript{-}null mice following O\textdegree-BG/TMZ treatment. **A**, at 6 days posttreatment, bone marrow from vehicle- and O\textdegree-BG/TMZ-treated mice was analyzed for the presence of human CD45\textsuperscript{+} and CD33\textsuperscript{+} cells via flow cytometry. **B**, on the basis of flow cytometry and bone marrow cell numbers, the number of human CD33\textsuperscript{‘} myeloid cells was determined. These data are representative examples of data from experiment I. Data are similar from experiment II, except that the level of overall human chimerism was lower in vehicle- and O\textdegree-BG/TMZ-treated mice. *, \(P < 0.0001\) for vehicle- versus drug-treated mice.
ranged from 2.7 to 19.5 μg/mL or 14.0 to 100 μmol/L, which is similar to TMZ peak levels obtained in adults (25). These TMZ dosing schedules have been incorporated into the standard-of-care protocols for glioblastoma (26). In the clonogenic assays as shown in Figure 1, we used clinically relevant doses of TMZ (25–100 μmol/L) that could be obtained in the plasma of patients and showed that human hematopoietic progenitor cells were significantly more sensitive than murine hematopoietic progenitors to O6-BG in combination with TMZ.

We used a O6-BG/TMZ regimen clinically known to be toxic to the bone marrow, confirmed that it did indeed block tumor growth of ectopic human xenografts, and importantly simulated therapy-mediated toxicity in the bone marrow. An O6-BG/TMZ treatment regimen was recently tested in phase II clinical trials in the absence of stem cell rescue (9). In that study, 5 of 32 patients with TMZ-resistant anaplastic glioma responded to O6-BG in combination with TMZ. For TMZ-resistant glioblastoma multiforme, only 1 of 34 patients exhibited a response to treatment. The reason for lack of response in most patients is likely a consequence of the necessity to reduce the levels of TMZ administered because of myelosuppression; a 25% reduction in TMZ dose was necessary in 48% of patients due to therapy-induced grade 4 neutropenia and thrombocytopenia. To increase doses of O6-BG/TMZ in these patients, myelosuppression will need to be overcome and this is an area of active investigation. Strategies that protect hematopoietic cells from high-dose O6-BG/TMZ such as viral vector–mediated gene transfer and expression of O6-BG–resistant mutant MGMT in autologous hematopoietic stem and progenitor cells (27), or strategies that incorporate stem cell rescue following administration of O6-BG/TMZ, may be the only treatment strategies to alleviate O6-BG/TMZ–associated bone marrow toxicity.

When NOD/SCID/γchainnull mice transplanted with human CD34+ cells were treated with 1 cycle of O6-BG/TMZ, significant differential toxicity of human versus mouse hematopoietic cells was observed. The reason for the differential sensitivities between mouse and human hematopoietic cells could be due, in part, to subtle differences in expression or in the amino acid structure of murine and human MGMT in the bone marrow cells. Although the active site of murine and human MGMT is identical (Pro-Cys-His-Arg), differences in the MGMT amino acid sequence are evident between the 2 species. Gerson and colleagues showed that, in contrast MGMT, murine MGMT has a leucine unit at residue 180. This amino acid residue is located just outside the active site of MGMT and correlated with increased resistance of murine MGMT to O6-BG (28).

A variety of human cancers and diseases are currently being modeled in immunodeficient mouse strains such as NOD/SCID (29). NOD/SCID mice transgenic for human stem cell factor, granulocyte-macrophage colony-stimulating factor, and interleukin-3 (30, 31), NOD/SCID/γchainnull (32–36), and Rag2−/−/γc−/− (39). Because of increased and consistent levels of human hematopoietic cell engraftment and an increased life span compared with NOD/SCID mice, NOD/SCID/γchainnull mice are beginning to replace NOD/SCID mice in studies that are testing transfer vector designs and strategies for hematopoietic gene therapy (38). In addition, development of preclinical humanized models that will allow for reconstitution of a mature human immune system will be instrumental in gaining a better understanding of in vivo human cell–mediated immune responses and for testing of novel immunotherapy or chemotherapy that target the tumor and its microenvironment. Shultz and colleagues recently shown that HLA-restricted human immune responses can now be studied in vivo by using NOD/SCID/γchainnull mice that have been engineered to constitutively express HLA class I heavy and light chains (40). The use of immunodeficient mouse models to study cancer progression and testing of anticancer therapies is still in its infancy. Integrated modeling approaches that investigate human tumor cell growth in mice with humanized bone marrow capable of producing mature human immune cells will be instrumental in understanding the interplay between tumor progression, the tumor microenvironment, and the hematopoietic and immune systems. For example, our research team recently showed that human circulating progenitor cells that are phenotypically and functionally distinct from circulating endothelial cells could promote growth of human melanoma xenografts in NOD/SCID and NOD/SCID/γchainnull mice (Mund, Case, and Pollok, unpublished observations; ref. 41).

The engrafted human hematopoietic CD34+ cells and progeny derived from the initial transplanted CD34+ cells interact, at least to some extent, with stromal cells and cytokines located in the murine bone marrow microenvironment because the transplanted human CD34+ cells differentiate into B-lymphoid and myeloid cells in vivo. Murine stromal feeder cells clearly interact with human hematopoietic cells because numerous studies have used stroma of murine origin for in vitro expansion of primitive human hematopoietic cells (42–45). In addition, it is important to note that in vivo models in which recapitulation of the complete spectrum of human hematopoiesis can be accomplished have been developed. For example, implantation of human fetal thymus and fetal liver into SCID mice provided a human microenvironment that allowed for differentiation of human hematopoietic cells into multiple cell lineages (46). An expansion of this model was carried out by Fraser and colleagues, in which SCID mice were transplanted subcutaneously with human fetal bone, thymus, and spleen fragments; this allowed for differentiation of human hematopoietic cells into multiple lineages and homing of cells to the thymus for further differentiation into mature T-cell subsets (47). More recently, it has been shown that human marrow mesenchymal stem/stromal cells (MSC) can organize a hematopoietic microenvironment with murine hematopoietic cells after transplantation into NOD/SCID mice (48). It is possible that the greater drug susceptibility of the engrafted human hematopoietic cells than that of the
mouse hematopoietic cells is that the mouse bone marrow microenvironment may not completely support human hematopoietic cell survival, particularly when under chemotherapy-mediated stress. However, in vitro clonogenic data also indicated striking differences in drug sensitivity of murine and human progenitor cells. Interactions between human MSC and hematopoietic stem and progenitor cells are important for survival and differentiation in vivo, for others have shown that cotransplantation of human MSC and CD34+ cells into NOD/SCID mice enhanced engraftment of human myeloid cells in the bone marrow (49). In addition, it is clear that the marrow microenvironment and MSC, as well as the hematopoietic stem and progenitor cells, are adversely affected by chemotherapy (50). At the present time, however, it is not known as to what extent the implantation of human MSC into the bone marrow of mice engrafted with human hematopoietic cells would increase or decrease the sensitivity of human hematopoietic cells to myelotoxic therapy. The humanized bone marrow model described here serves as a starting point for these types of investigations. Our data indicate that differential sensitivities of mouse versus hematopoietic cells to O6-BG in combination with TMZ exist in vitro (Fig. 1) and that these differences in drug sensitivity could be recapitulated in the in vivo environment (Table 1 and Figs. 3–5). The humanized bone marrow model described in our study could be used on a much larger scale for drug and regimen screening because only transplantation of reasonable numbers of human CD34+ cells into sublethally irradiated NOD/SCID/γcnull mice is required and confirmation of human engraftment in the bone marrow can be done by analyzing a small sample of PB from the transplanted animals.

The humanized bone marrow mouse model described here represents a feasible and physiologically relevant tool in which to determine if a balance between treatment efficacy and toxicity is achievable. In contrast to many other immunodeficient mouse strains, previous studies have shown that NOD/SCID/γcnull mice can be readily and consistently reconstituted with human hematopoietic cells that have the capacity to differentiate into various blood cell lineages (6). In addition, we have found that human tumors grow consistently in NOD/SCID/γcnull mice and represent a reliable model for testing anticancer strategies. In future studies, it will be essential to utilize orthotopic xenografts when new therapeutic regimens are tested because the microenvironment in which the tumor resides could affect tumor sensitivity to treatment (51). Integration of humanized hematopoietic and tumor models may be a way for anticancer therapies to be tested earlier in the drug development process, which will ultimately lead to more efficient translation of therapies into clinical practice. Although this model has the potential to be used as an in vivo screening tool that can aid in testing and refinement of treatment regimens, its validation will require testing of an additional number of compounds and regimens to establish its predictive value. We are currently in the process of organizing a consortium of scientists to evaluate a broader set of therapeutics to determine the predictive clinical value of this modeling approach.

Disclosure of Potential Conflicts of Interest

W.S. Goebel, medical director of General BioTechnology, LLC, The Genesis Bank, LLC, and Renovosyne, LLC, the other authors declared no potential conflicts of interest.

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