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ORIGINAL ARTICLE

In vitro comparison of O4-benzylfolate modulated, BCNU-induced toxicity in human bone marrow using CFU-GM and tumor cell lines

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Abstract 2-Amino-O4-benzylpteridine derivatives inactivate the human DNA repair protein O6-alkylguanine-DNA alkyltransferase and have been tested as modulators of alkylating agent chemotherapy. Recently, the therapeutic potential of O4-benzylfolate (O4BF) in modulating bischloroethylnitrosourea (BCNU) toxicity was demonstrated in vitro using human HT29 and KB tumor lines. The current studies replicated the previous findings in HT29 and KB cells using ATP as an endpoint. However, the effective treatment conditions were severely toxic to human neutrophil progenitors called CFU-granulocyte/macrophage (CFU-GM), which could not tolerate ≥40 μM BCNU at any O4BF concentration. A lower BCNU concentration (10 μM) in combination with O4BF (2–100 μM) was only moderately tumoricidal. To screen for conditions tolerated by CFU-GM, bone marrow (BM) cells were pre-incubated (5 h) with O4BF, co-treated with O4BF and BCNU (42 h), washed, and plated to quantify CFU-GM survival. O4BF at 2 or 5 μM progressively lowered the inhibitory concentrations (ICs) for BCNU, but further increases in O4BF concentrations did not. Increasing O4BF concentrations with constant BCNU (10 μM) under the same prolonged exposure as in the human marrow study achieved only modest tumoricidal effects. In summary, the unexpected finding that normal BM cells are impacted by an agent developed to target malignant tissue refutes speculation that normal β-folate receptor expressing hematopoietic cells will be spared. Further, the validated IC_{90} endpoint from the huCFU-GM assay has provided a reference point for judging the potential therapeutic effectiveness of this investigational combination in man using in vitro assays.

Keywords CFU-GM · Human bone marrow · KB and HT29 tumor cells · Neutropenia · O4-benzylfolate

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Introduction

During the past three decades, the chloroethylating agent bischloroethylnitrosourea (BCNU), a member of the nitrosourea class of anti-cancer drugs, has been a commonly used drug to treat patients with malignant gliomas. However, many tumors are resistant to BCNU; therefore, it has had only limited success in such tissues [25, 30]. To maximize the efficacy of the nitrosourea class of anti-cancer drugs, the potential for combination therapies is being evaluated in the treatment of cancer patients. Studies have demonstrated that the DNA repair protein O6-alkylguanine-DNA alkyltransferase (AGT) is responsible for repair mechanisms involved in adduct (such as methyl- or chloroethyl-) removal from the O6-position of guanine [29, 39]. A hindrance to effective chemotherapy is the high expression of AGT in tumor cells, conferring resistance to alkylnitrosourea-induced damage. Subsequently, a number of studies have been conducted that have confirmed the increased efficacy of BCNU with combination of AGT inhibitors [2, 4–11, 17–19, 24, 26–28, 31, 41, 50, 51]. Of the AGT inhibitors, O6-benzylguanine (O6BG) and O6-(4-bromothenyl)guanine have progressed to clinical trials [1, 16, 20, 21, 44]. However, poor solubility stimulated the development of

numerous analogs with more favorable pharmacological properties [3, 24, 40, 47].

Recently, the analog O4-benzylfolate (O4BF) has attracted interest due to its high water solubility and potent anti-AGT activity [16, 33]. The inactivation of wild-type human AGT by O4BF was shown to be superior (approximately 30-fold greater) than that of O6BG, with a reported ED50 in the nanomolar range. O4BF was also found to be effective against the P140K mutant alkyltransferase. Its greater effectiveness as an AGT inactivator has been attributed to favorable displacement and steric conditions, and it has been shown to enhance BCNU tumoricidal activity, especially in tumor cell lines that differentially express active α-folate receptors in folate-free medium [24, 33]. As studies examining O4BF's therapeutic potential in combination with BCNU progress, potential adverse effects are also being explored. Among these are studies indicating that β-folate receptors are expressed in human bone marrow (BM) and $CD34^+$ hematopoietic cells but that folates are transported only in leukemic but not normal bone marrow [14, 34, 45], suggesting that O4BF would spare the hematopoietic progenitors responsible for producing myeloid blood cells.

The colony-forming assay is a valuable in vitro assay that has been used since the 1970s to assess human hematotoxicity [12, 13, 32, 36, 38, 48, 49]. In particular, the colony-forming unit, granulocyte/macrophage (CFU-GM), preclinical screening assay has been a useful tool in predicting adverse effects of drugs that would cause neutropenia in humans [22, 35]. Although IC_{50} values are traditionally used for comparative purposes, this endpoint was not found to be clinically useful for the CFU-GM assay. An in vivo–in vitro study relating the clinical myelotoxicity of 9-methoxypryazoloacridine to the inhibition of myeloid progenitors in vitro indicated that the drug exposure levels that inhibit neutrophil progenitors by 90% in vitro and decrease ANC by 90% in vivo were the same [37]. A subsequent study confirmed the importance of the IC_{90} value when species differences in sensitivity to the camptothecin class of compounds were predicted using this metric [15]. In short, the success and reproducibility of the assay has led to collaborative efforts that established a standard operating procedure and subsequent validation of the assay for acceptance by the European Center for Validation of Alternative Methods (ECVAM) [32, 35, 38, 42, 43].

In this report, we have tested the combination of O4BF with BCNU using the CFU-GM assay to address concerns about dose-limiting toxicities (DLT), namely, severe neutropenia under conditions that would be considered clinically relevant. Given that a folate transport system has been exploited for targeted delivery of the AGT inhibitor to tumor cells that express high levels of α-folate receptors, and that the β-folate receptor does not bind folate in normal, mature neutrophils [34, 45], the current study was undertaken to confirm that the possible presence of the β-folate receptor on hematopoietic cells is not a concern for the safety of the O4BF/BCNU treatment regimen. In addition, the current study seeks to demonstrate selectivity of the combination of O4BF with BCNU for human tumor cells compared to the huCFU-GM progenitor in bone marrow.

Materials and methods

Materials

O4BF (NSC742482) and BCNU (NSC409962) were obtained from the NCI repository (Rockville, MD). Dehydrated alcohol USP (EtOH) was purchased from American Regent Inc. (Shirley, NY). The ATPlite assay kit was purchased from Perkin Elmer (Waltham, MA).

Human donor bone marrow

The CFU-GM clonogenic assay was conducted using freshly collected, human bone marrow cells (Lonza-Biowhittaker, Walkersville, MD). All human donors are healthy, pre-screened individuals and the tissue samples were handled in accordance with the NIH Bloodborne Pathogens SOP for NCI-Frederick, as well as in accordance with the institutional IBC guidelines. Bone marrow was collected from five donors (4 occasions) and from three donors (1 occasion) for long- and short-term treatment studies, respectively. Bone marrow was processed and the purified mononuclear cells (MNCs) were shipped to our laboratory the following day.

CFU-GM assay

Long-duration treatment of CFU-GM

Upon arrival, the cells were gently pelleted and the transport media removed. The cells were then resuspended in 5 ml of Plasma-Lyte A USP (Baxter Healthcare, Deerfield, IL), mixed well and treated with 2.5 µl/ml Pulmozyme (Genentech Inc., South San Francisco, CA). After a 10-min room temperature incubation, the cells were layered over 5 ml Ficoll-Paque PLUS (Stem Cell Technologies, Vancouver, BC) and centrifuged for 30 min at 1,500 relative centrifugal force to enrich the viable MNCs population. The buffy layer containing the MNCs was collected, washed in 14 ml Plasma-Lyte A USP, and finally resuspended in 10 ml IMDM (Stem Cell Technologies, Vancouver, BC). Cell counts were performed using a hemacytometer. For each O4BF concentration, a minimal volume of cell suspension yielding 110,000 cells/ml was added to 54 ml complete medium in 125-ml storage bottles

(Corning, Lowell, MA) containing IMDM, 20% fetal bovine serum (Lonza, Walkersville, MD), 100 ng/ml gentamicin (Abraxis, Schaumburg, IL), and 10 ng/ml Leukine sargramostin GM-CSF (Berlex, Seattle, WA). O4BF was solubilized in IMDM to make a 10× stock drug solution. The 10× stock drug solution was diluted into 15-ml conical vials (Falcon, Franklin Lakes, NJ) in IMDM to prepare the 10× test concentrations, and 6 ml of each test concentration was were added to the corresponding cell suspension. Cells were mixed well and 10-ml aliquots dispensed in T-25 ml tissue culture-treated, vented flasks (Corning, Lowell, MA). The flasks were placed upright in the incubator for 5 h at 37°C with 5% CO_2. BCNU was solubilized in EtOH at 2,000×. After incubation, 5 µl BCNU was added to each of the flasks, mixed and returned to the incubator for 40 h.

Short-duration treatment of CFU-GM

The purified MNCs from each donor were suspended in 40 ml of complete medium and were split in two T-75 ml vented tissue culture flasks (Corning, Lowell, MA). The flasks were placed in the incubator for 24 h to provide time to respond to cytokine stimulation. The cells from one of each donor flask were counted using a hemacytometer. For each O4BF concentration, a minimal volume of cells to yield 82,500 per ml was added to 36 ml complete medium in 50-ml conical vials (Falcon, Franklin Lakes, NJ). O4BF was solubilized in IMDM to make a 10× stock drug. The 10× stock drug was further diluted in 15-ml conical vials in IMDM to obtain the final testing concentrations, and for each treatment, 4 ml of treatment media was added to the cell suspensions. Cells were mixed well and 10-ml aliquots dispensed in T-25 ml tissue culture-treated, vented flasks (Corning, Lowell, MA). The flasks were placed upright in the incubator for 2 h at 37°C with 5% CO_2. BCNU was solubilized in EtOH at 2,000×, 5 µl BCNU was added to each of the flasks, mixed and returned to the incubator for 2 h. After the incubation period, the contents of the flasks were transferred to 15-ml conical vials, centrifuged, and treatment media removed. 10 ml of complete medium with O4BF was added to the vials, mixed well and, the contents transferred to new T-25 ml flasks and incubated for 16 h.

Plating of treated cells for CFU-GM

Following incubation, the contents of the flasks were then transferred to 15-ml conical vials, centrifuged, and the treatment medium removed. To each vial of pelleted cells, 8.5 ml of complete medium was added and the vials placed in a 37°C waterbath. After warming, 1.5 ml of 2.5% SeaPlaque Agarose (Lonza-Biowhittaker, Walkersville, MD) in water was added, vortexed well, and 2 ml was plated in triplicate in 6-well plates containing a pre-gelled, 2 ml underlayer of IMDM, Fetal Bovine Serum and 2.5% SeaPlaque Agarose per well. Each well contained 200,000 human donor MMC. The plates were placed at 4°C until completely gelled and placed in the incubator at 37°C with 5% CO_2 for 14 days. After 14 days, the colonies were counted and IC_{90} values calculated (by linear regression), using the vehicle control as a reference.

HT29 and KB cytotoxicity assay

Two NCI60 cell lines, HT29 and KB (NCI-Frederick repository, Frederick, MD), were cultured in RPMI 1640 plus 2 mM GlutaMAX (GIBCO-Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Lonza-Biowhittaker, Walkersville, MD), and maintained in T75 culture flasks (Corning, Lowell, MA) at 37°C in a humidified atmosphere of 5% CO_2. For harvesting and use in experiments, cells were trypsinized with 0.25% Trypsin EDTA solution (Sigma, St. Louis, MO) and plated into 96-well solid black plates with clear bottoms (Corning Inc., Corning, NY) at 2,000–5,000 (HT-29 cells) or 5,000–10,000 cells (KB cells) per ml in 100 µl of medium and allowed 24 h to attach to plate and recover. The cell line specific plating density was increased in later experiments (based on growth characteristics) to maximize signal to noise ratio. After short- or long-duration treatment (see below), relative ATP levels were determined in accordance with the manufacturer's protocol. The 96-well plates were taken from the incubators and allowed to equilibrate for 30 min. The medium was aspirated from wells and 100 µl of PBS (Lonza-Biowhittaker, Walkersville, MD) was added. Next, 100 µl of ATPlite substrate solution was added to the wells and the plate was shaken vigorously for 2 min. Relative luminescence (RLU) was measured after 5–10 min on a TECAN GENios Pro plate reader (Research Triangle Park, NC).

Short-duration treatment for cytotoxicity assay

For the short-duration exposure, cells were pretreated with O4BF (0, 2, 5, 10, 20, 40, 80 or 100 µM) prepared in medium for 2 h prior to the addition of BCNU 10 or 40 µM (BCNU prepared as previously described [33]) for 2 h. The medium was then aspirated and replaced with medium containing only respective O4BF concentrations and incubated for an additional 16 h. Medium was once again aspirated and replaced with fresh medium devoid of treatment and cultured for 4 days before being assayed for ATP content.

Long-duration treatment for cytotoxicity assay

For the longer duration exposure, the cells were pretreated for 5 h with O4BF, 2–50 µM, prior to the addition of

10 μM BCNU, for an additional 42 h. After 42 h, the medium was aspirated and replaced with medium without drugs and cultured for 4 days before being assayed for ATP content.

Results

Previously reported, effective tumoricidal conditions are not clinically feasible

To establish a comparison of the clonogenic assay previously used to report the enhanced tumoricidal effects of the O4BF/BCNU combination with the ATPlite assay, the first experiment replicated the treatment conditions used previously [33]. However, the purpose of substituting the ATP assay for the clonogenic assay used by Nelson et al. [33] was to shorten and simplify it, so the length of time following treatment removal and quantitation of the assay was reduced from 8 to 4 days. The ATPlite assay was conducted on human HT-29 (colon carcinoma) and KB (nasopharynx carcinoma) cell lines, known to express low and high levels (respectively) of the α-folate receptor required for O4BF transport. The purpose of this work was also to establish the baseline effects of O4BF combination with BCNU at the same concentrations previously reported using a clonogenic assay. The results of the assay were comparable to those previously reported in that HT29 and KB cells had a similar pattern of response to the combination of 25 μM O4BF with 40 μM BCNU (Fig. 1). It was concluded that the ATP assay was a good substitute for the lengthier and more burdensome clonogenic assay, and so the ATPlite assay was also used in subsequent studies that assessed the efficacy of combination treatment regimens.

The preliminary work assessing the tumoricidal effect of 40 μM BCNU co-applied with 10 and 25 μM O4BF prompted our laboratory to assess the potentially toxic effects on hematopoietic progenitor cells in human bone marrow. Using the same treatment concentrations and duration, the CFU-GM assay was run and the colonies scored after the requisite 14 days in culture (post-treatment). Vehicle control colony counts (± standard deviation) were as follows for the three human donors: 90 ± 10, 107 ± 7, 139 ± 15. It was found that 40 μM BCNU alone caused an ~80% drop in GM colony formation, and the combination of even the lowest designated concentration of O4BF (2 μM) resulted in well over 90% colony loss, the threshold predictive of severe neutropenia in humans (Fig. 2).

With the data indicating that lower concentrations of BCNU are necessary for the safe combination of O4BF, another efficacy study using HT29 and KB cells was performed using a lower concentration of BCNU (10 μM) but using higher O4BF concentrations spanning from 2 to 100 μM. It was found that the amount of enhanced efficacy O4BF affords is minimal in both HT29 and KB cell lines. The results show that the KB cell line (known to express high levels of the α-folate receptor) was impacted greater than the HT29 cell line, which is known to express only a moderate level of the α-folate receptor (Fig. 3).

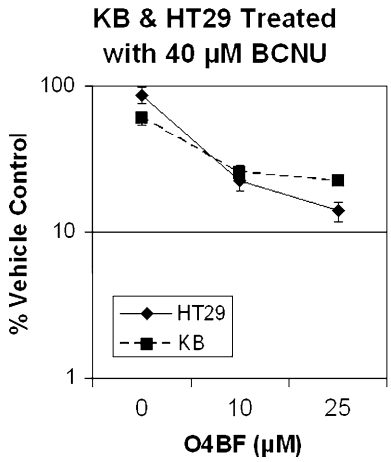

Fig. 1 HT29 and KB cell response to O4BF and BCNU combination, using the ATPlite assay. "Short" exposure conditions were the same as previously reported [33]. Cells were pretreated with O4BF for 2 h, then treated with both O4BF and BCNU for 2 h, followed by 16 h of O4BF alone. Results indicate a similar profile of response when compared to previously reported clonogenic assays using the same cell lines. ATPlite raw data ($n = 4$–5 experiments with 4–8 replicates per control or treatment group per experiment) were recorded as RLU and sample values were calculated as percentage of vehicle control. *Error bars* indicate standard deviation of the mean

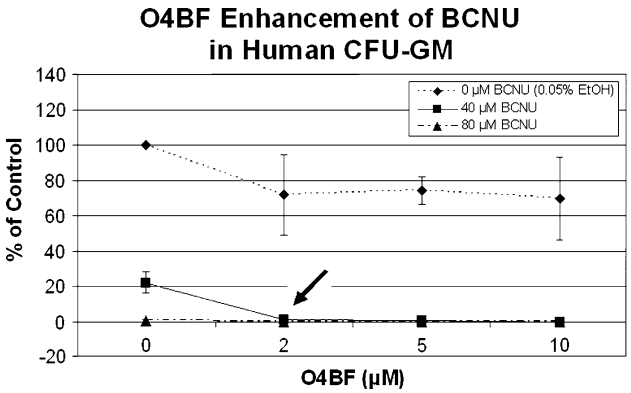

Fig. 2 Human CFU-GM using previously reported "short" exposure conditions [33]. Greater than or equal to 40 μM BCNU with 2 μM (or more) O4BF predicted conditions unsafe for administration in humans. *Arrow* indicates lowest concentration of O4BF applied, resulting in 98% colony loss. Data are an average of three experiments using human donor MMC. *Error bars* on data *points* indicate standard deviation

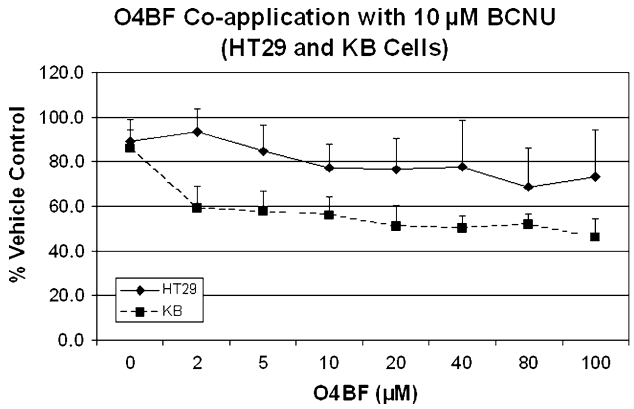

Fig. 3 HT29 and KB cells treated with 10 µM BCNU show low toxicity even with high concentrations of O4BF. "Short" exposure conditions (see "Materials and methods") were the same as previously reported [33]. ATPlite raw data ($n = 4$ experiments with 4–8 replicates per control or treatment group per experiment) were recorded as RLU and sample values were calculated as percentage of vehicle control. *Error bars* indicate standard deviation of the mean

Derivation of clinically feasible BCNU and O4BF co-treatment using refined conditions

The attempts to find concentrations of BCNU and O4BF that function in a tumoricidal manner while also constituting safe levels were unsuccessful using the previously reported treatment scheme. Therefore, a CFU-GM study was conducted that utilized a longer treatment period with a range of BCNU and O4BF concentrations to determine what would be therapeutically feasible. Bone marrow cells (in suspension with medium containing GM-CSF) were first exposed to O4BF (0, 2, 5, 10, 20 µM) for a period of 5 h. followed by the addition of BCNU (0, 10, 20, and 40 µM) for an additional 42 h, which allowed several cell divisions to be impacted by both BCNU and the inhibited AGT repair mechanism. Following the exposure period, cells were washed of treatment and plated on the semi-solid agarose for colony growth over a period of 14 days. Vehicle control colony counts were as follows for the five donors tested: 289 ± 29, 700 ± 20, 252 ± 11, 325 ± 40, 246 ± 6. Following the quantitation of colony growth, the IC_{90} values were calculated using linear regression and tabulated (Table 1).

Results from this lower concentration but longer exposure study indicate an IC_{90} value calculated at approximately 10 µM BCNU, with maximal toxicity seen at 10 µM O4BF combination, using BM from this set of five human donors (Fig. 4).

Lack of relevant effect using clinically feasible conditions

Given that 10 µM was found to be the maximal BCNU concentration possible with combination of O4BF, the tumoricidal efficacy under these clinically feasible conditions was evaluated. Again, HT29 and KB cells were exposed to BCNU and O4BF but using the modified treatment conditions described above for the CFU-GM assay. It was found that both HT29 and KB cells reacted to the treatment as expected, with the higher folate receptor-expressing KB cell line being more susceptible to the effects of BCNU and O4BF co-treatment. However, the enhanced tumoricidal effects afforded by the combination of O4BF were only marginally effective (Fig. 5).

In summary, we have used a simple ATP assay to measure comparable cytotoxic responses previously reported using a clonogenic assay when HT29 and KB tumor cell lines were co-exposed to BCNU and the AGT inhibitor O4BF. When these treatment conditions were implemented using the CFU-GM assay, it was predicted that the length of exposure and concentrations of BCNU and O4BF co-applied would cause severe neutropenia in humans. Repeating the assay in the tumor cells, but using a lower concentration

Table 1 IC_{90} values for O4BF-modulated, BCNU-induced human MMC toxicity

O4BF (µM)	Human donor					Range	Average ± SD
	2	3	4a	4b	5		
0	39.5	33.8	38	24.7	34.2	24.7–39.5	34 ± 5.7
2	17.5	16.2	25	13.8	13.9	13.8–25.0	17.3 ± 4.6
5	15.1	12.6	8.7	15.3	12.4	8.7–15.3	12.8 ± 2.7
10	12.4	10.5	8.6	9.9	11.3	8.6–12.4	10.5 ± 1.4
20	9.8	8.8	20.2	9.6	9.4	8.8–20.2	11.6 ± 4.9

MMC were plated following a 5-h preincubation with O4BF and subsequent ~42-h co-incubation with BCNU (0, 10, 20, and 40 µM) before plating

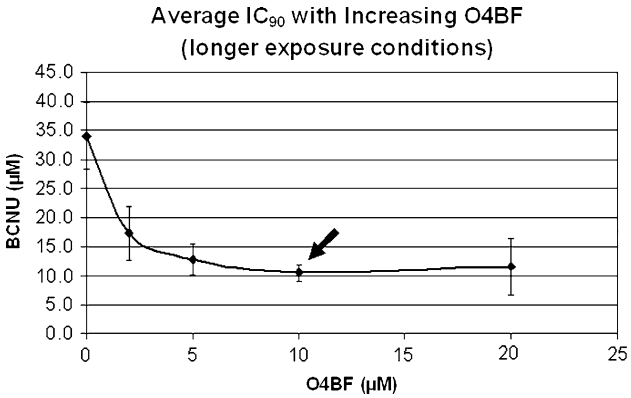

Fig. 4 Human CFU-GM study designed to establish clinically feasible BCNU and O4BF treatment conditions. "Long" exposure conditions consisted of cells being pretreated for 5 h with O4BF before the addition of BCNU for an additional 42 h, washing, and plating for colony growth. The *arrow* indicates the maximally tolerated BCNU concentration when co-applied with O4BF on human BM cells. Data are an average of four experiments (± standard deviation) using human MMC from five donors

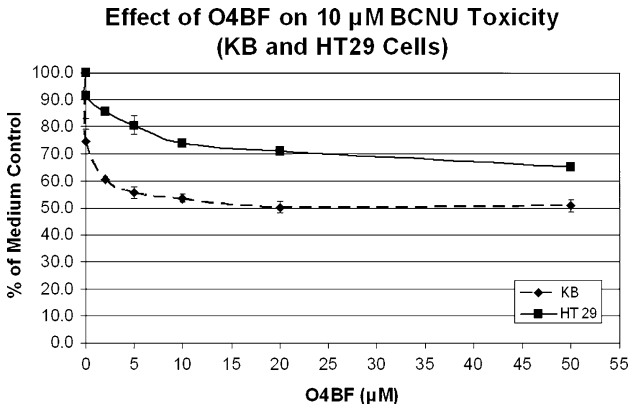

Fig. 5 HT29 and KB cell line exposure to BCNU and O4BF under tolerable ("Long exposure") conditions. The enhancement of BCNU toxicity was only marginal with the combination of O4BF. ATPlite raw data (n = 4–5 experiments with 4–8 replicates per control or treatment group per experiment) were recorded as RLU and sample values were calculated as percentage of vehicle control. *Error bars* indicate standard deviation of the mean

of 10 µM BCNU in combination with higher O4BF concentrations, it was found that a moderate increase (over BCNU alone) of toxicity was exerted, but a more severe effect was desired. Seeking a viable co-treatment alternative, treatment schemes were tested where IC_{90} values were determined using an array of BCNU and O4BF concentration combinations in CFU-GM assays using human BM. Results indicated an acceptable concentration of 10 µM BCNU in combination with 10 µM (or greater) O4BF when longer exposure times are used. Using this same (predictably feasible) treatment scheme, HT29 and KB cells were challenged to determine the tumorigenic efficacy in the HT29 and KB folate receptor-expressing cells. It was found that O4BF concentrations beyond 20 µM (with concentrations up to 100 µM) did not enhance BCNU's capability in killing malignant cancer cells. The same characteristic O4BF plateau effect was again seen, as with previous combination studies.

Discussion

The rising interest in combination therapies has spurred the re-evaluation of traditional chemotherapeutics co-applied with drugs designed to enhance their effect. BCNU has been an effective anti-cancer drug in some malignancies, so the screening of compounds designed to potentiate its ability to cause DNA adduct formation and subsequent tumor cell death is ongoing. It is known that many tumor cells express high levels of the DNA repair protein AGT and that many tumor cells also express a high level of α-folate receptors/transporters. These two tumor cell properties are being exploited in the design of effective combination therapies. O6BG is an effective AGT inhibitor but its undesirable pharmaceutical properties (short-lived effects and poor solubility) led to the development of O4BF, a potent and highly water-soluble analog that enters cells via folate receptor/transporter system(s).

For O4BF to be a viable candidate for development as a BCNU modulator, it must be able to increase BCNU's effectiveness against the tumor target at concentrations that can be tolerated by likely dose-limiting tissues like the bone marrow for BCNU therapy. Previous in vitro efficacy studies identified effective tumoricidal concentrations of BCNU in combination with O4BF. It was determined that 20–25 µM O4BF in combination with 40 µM BCNU caused over 90% decline in clonogenic colony formation in the HT29 and KB tumor cell lines. There was some concern that β-folate receptors already shown to be present in human bone marrow specimens might be active transporters of folates in hematopoietic cells like CFU-GM. Our results using BM from multiple donors and in vitro treatment conditions previously reported (40 µM BCNU) indicated that even a modest concentration (2 µM) of O4BF would be predicted to cause severe neutropenia in a patient, i.e., the in vitro myelotoxicity exceed the IC_{90} value. For this reason we chose to explore whether longer exposures of lower concentrations of BCNU would be better tolerated by CFU-GM, so concentrations of O4BF of 20 µM or more could be reached. The new treatment conditions were found to be tolerable and the toxic effect of O4BF modulation of BCNU was found to plateau at 10 µM. This revised treatment scheme was then tested on HT29 and KB cells to determine the tumoricidal efficacy using the ATPlite assay. Results (Fig. 1) demonstrated similar findings as previously reported using the clonogenic assay (under the same treatment conditions) and suggests that the ATPlite assay could possibly be used as a substitute to more rapidly evaluate tumoricidal effects of compounds in vitro. As expected, the enhancing effect of O4BF also had a plateau/ceiling effect, and increasing its concentrations up to 50 µM did not enhance BCNU's cytotoxicity (Fig. 5). From these data, we concluded that O4BF only modestly increased the tumoricidal effects of BCNU under conditions tolerated by human CFU-GM, and neither condition improved the selectivity of BCNU for tumor over CFU-GM.

The study reported here indicates that the use of 40 µM BCNU alone was tolerable in CFU-GM (using the IC_{90} cutoff as a threshold for predicted neutropenia in humans), and this supports previously reported data [49]. However, even the lowest concentration of O4BF co-applied with 40 µM BCNU resulted in 98% colony loss (Fig. 2), which would be predicted to cause severe neutropenia in vivo. This evidence undermines previous reports suggesting that the β-folate receptor expressed in normal tissues (e.g., hematopoietic cells) is unable to bind folate, therefore

sparing them from the adverse effects of folate receptor targeting chemotherapeutics. With hematopoietic tissues exhibiting sensitivity to O4BF/BCNU combinations, the reality of a BM DLT must be considered when determining a feasible dosing regimen of combination therapy. Perhaps the mechanism by which O4BF interacts with human BM treated with BCNU can be better understood, so that different O4BF analogs can be developed that are selective for the α-folate receptor system present in tumor but not in BM.

The development of potential anti-cancer therapies will always involve the requirement for efficacy at exposures that are safely tolerated. In the case of O4BF, an AGT inhibitor designed to be transported by folate receptors, the potential impact on normal tissues also expressing folate transporters is of concern. Current literature describes the distribution of human folate receptors that are involved in transport (α and β subtypes) [14, 23, 34, 45]. With narrow tissue specificity and overexpression of α-folate receptors in malignant tissues, this receptor system has become a drug target to selectively target cancerous tissue. The β-folate receptor is expressed in normal $CD34^+$ progenitor cells in the BM, in mature neutrophils, and placenta, but in relatively low or insignificant levels in other tissues. Due to the different ligand-binding characteristics, different tissue specificities, and selective regulation in malignant tissues, it has been suggested the α and β folate receptor systems could be exploited in designing anti-folate chemotherapeutics [46]. Studies have demonstrated that only leukemic cells expressing the β-folate receptor are able to bind folate [34, 45], suggesting normal tissues expressing this receptor subtype may be spared from folate receptor-targeting drugs. In the study reported here, we describe a lack of selectivity of the BCNU/O4BF combination for tumor cells over BM. This finding refutes speculation that non-malignant BM cells are spared from the adverse effects of folate receptor-targeting agents, and suggests that at least hematopoietic progenitors in the marrow use the folate transport system. This is perhaps not surprising given the myelosuppressive effects of anti-folates like methotrexate. It should be noted that the development of folate-based AGT-targeting agents has coincided with the detection and ongoing functional characterization of the β- and other folate receptor subtypes, making it difficult to explain phenomenon such as reported lack of folate binding to β-folate receptors in normal tissues in conjunction with our findings. With regards to the $CD34^+$ cell population, it is known that cytokine stimulation is required for functionality of mature cells. Reddy et al. found that overnight stimulation with IL-6 and stem cell factor, and lengthier exposures to cytokines generally, may be necessary for full folate receptor functionality and for conducting folate-binding studies. Pan et al. also reported lack of FITC-folate binding in granulocytes, but it is unclear whether any cytokines required for full functionality were used when preparing cells for experimentation. Nonetheless, the results of the current study show that O4BF in combination with BCNU adversely impacted normal BM cells and that folate receptors in these cells must be evaluated when designing safe folate receptor-targeting chemotherapeutics.

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