

# O<sup>6</sup>-Methylguanine-DNA Methyltransferase in Tumors and Cells of the Oligodendrocyte Lineage

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**ABSTRACT: Background:** Oligodendrogliomas respond to nitrosourea-based chemotherapy and are induced in rats following transplacental exposure to ethylnitrosourea, observations suggesting that neoplastic and normal cells of the oligodendrocyte lineage are “sensitive” to nitrosoureas. Nitrosoureas alkylate DNA at O<sup>6</sup>-guanine with repair mediated by O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT). The cytotoxic and carcinogenic properties of the nitrosoureas appear related to MGMT activity. **Methods:** To explore why oligodendrogliomas respond to chemotherapy, we measured MGMT activity in five chemosensitive human oligodendrogliomas and in rat oligodendrocyte lineage cells. We also measured MGMT activity in rat astrocytes and compared the cytotoxic effects of carmustine (BCNU) on oligodendrocyte lineage cells and astrocytes. **Results:** Low levels of MGMT activity were found in five of five human oligodendrogliomas. Cultures of neonatal rat glia enriched for oligodendrocyte lineage cells also had low levels of MGMT activity, approximately one-third that found in astrocytes ( $p < 0.02$ ), and oligodendrocyte lineage cells were more sensitive to BCNU than astrocytes. **Conclusions:** Low MGMT activity may contribute to the chemosensitivity of some human oligodendrogliomas and rat oligodendrocyte lineage cells also have low levels. If drug resistance mechanisms in tumors reflect the biochemical properties of their cells of origin, then normal glia may serve as a laboratory substitute for human glioma.

**RÉSUMÉ: La O<sup>6</sup>-méthylguanine ADN méthyltransférase dans les tumeurs et les cellules de la lignée oligodendrocytaire. Introduction:** Les oligodendrogliomes répondent à la chimiothérapie par les produits à base de nitrosourées et sont induits chez le rat à la suite d’une exposition transplacentaire à l’éthylnitrosourée. Ces observations suggèrent que les cellules tumorales et les cellules normales de la lignée oligodendrocytaire sont sensibles aux nitrosourées. L’ADN est alkylé par les nitrosourées au niveau de l’O<sup>6</sup>-guanine et la réparation est médiée par l’O<sup>6</sup>-méthylguanine ADN méthyltransférase (MGMT). Les propriétés cytotoxiques et carcinogéniques des nitrosourées semblent reliées à l’activité de la MGMT. **Méthodes:** Nous avons mesuré l’activité de la MGMT dans cinq oligodendrogliomes humains chimiosensibles et dans des cellules de la lignée oligodendrocytaire de rat afin d’explorer pourquoi les oligodendrogliomes répondent à la chimiothérapie. Nous avons également mesuré l’activité de la MGMT dans les astrocytes de rat et comparé les effets cytotoxiques de la carmustine (BCNU) sur les cellules de la lignée oligodendrocytaire et sur les astrocytes. **Résultats:** Nous avons trouvé des niveaux bas d’activité de la MGMT dans cinq oligodendrogliomes humains sur cinq. Des cultures de névroglie de rat nouveau-né enrichies en cellules de la lignée oligodendrocytaire avaient également des niveaux bas d’activité de la MGMT, à peu près au tiers de celui des astrocytes ( $p < 0.02$ ), et les cellules de la lignée oligodendrocytaire étaient plus sensibles au BCNU que les astrocytes. **Conclusions:** Une activité basse de la MGMT peut contribuer à la chimiosensibilité de certains oligodendrogliomes humains et les cellules de rat de la lignée oligodendrocytaire ont également des niveaux bas. Si les mécanismes de chimiorésistance des tumeurs reflètent les propriétés biochimiques de leurs cellules d’origine, la névroglie normale pourrait servir en laboratoire de substitut au gliome humain.

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Oligodendrogliomas respond predictably to nitrosourea-based chemotherapy, especially PCV (procarbazine, CCNU (lomustine) and vincristine),<sup>1-3</sup> and are preferentially induced in rats following transplacental exposure to ethylnitrosourea.<sup>4,5</sup> These observations suggest that neoplastic and normal cells of the oligodendrocyte lineage are “sensitive” to the nitrosourea family of alkylating drugs. The biochemical basis of this special vulnerability is unknown.

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The nitrosoureas alkylate DNA at O<sup>6</sup>-guanine and repair at this site is mediated by O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT). The cytotoxic and carcinogenic properties of the nitrosoureas appear related to MGMT activity; cells rendered MGMT-positive by DNA transfection become resistant to the nitrosoureas,<sup>6,7</sup> resistant cells depleted of MGMT by pretreatment with O<sup>6</sup>-benzylguanine are sensitized to the nitrosoureas<sup>8,9</sup> and cells susceptible to neoplastic transformation by nitrosoureas express low levels of MGMT.<sup>10,11</sup> Moreover, procarbazine, the other major component of the PCV regimen, also produces cytotoxicity by alkyl substitution at O<sup>6</sup>-guanine.<sup>12</sup> To explore why gliomas with oligodendroglial differentiation might be chemosensitive, we measured MGMT activity in human oligodendrogliomas and rat oligodendrocyte lineage cells. We also measured MGMT activity in rat astrocytes and compared the cytotoxic effects of carmustine (BCNU) on oligodendrocyte lineage cells and astrocytes.

## MATERIALS AND METHODS

**Tumor Specimens** Oligodendroglioma tissue from two brain tumor banks was analyzed for MGMT activity; four tumors were anaplastic, one was enhancing but non-anaplastic, and all were chemosensitive. Each specimen had been quick-frozen in the operating room and stored at minus 70°C or in liquid nitrogen.

**Materials** Culture media, fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin were obtained from GIBCO Laboratories (Burlington, ON). All other chemicals, including 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma Chemicals (St. Louis, MO). A2B5 antibody was obtained from the American Type Culture Collection, antibody to galactocerebroside (GC) from Boehringer Mannheim (Laval, PQ), and antibody to glial fibrillary acidic protein (GFAP) from Dimension Laboratories Inc (Mississauga, ON). Rhodamine- and fluorescein-conjugated secondary antibodies were also obtained from Dimension Laboratories Inc (Mississauga, ON). BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) was purchased from Bristol Laboratories of Canada (Montreal, PQ), and [<sup>3</sup>H]-methyl-nitrosourea from Amersham Corporation (Oakville, ON).

**Glial Cultures** Two populations of glial cells, oligodendrocyte lineage enriched and astrocyte enriched, were isolated from newborn rat cerebrums using a method modified from McCarthy and de Vellis.<sup>13</sup> Cerebral hemispheres were isolated from unanesthetized Sprague-Dawley neonates and dissected free of meninges and blood vessels. A single cell suspension was prepared by a combination of mechanical and enzymatic dissociation (0.025% trypsin). Cells were seeded into polylysine-coated 80 cm<sup>2</sup> flasks (2x10<sup>7</sup> cells/flask) and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 50 µg/ml transferrin, 5 µg/ml insulin, 30 nM selenium, 30 nM triiodothyronine and 50 U/ml penicillin/streptomycin. After five days the FBS concentration was reduced to 1% to enhance oligodendrocyte differentiation.<sup>14</sup> At day nine, oligodendrocyte lineage cells were separated from astrocytes by overnight rotary shaking in DMEM containing 10% FBS, L-glutamine and 5 mM leucine methyl ester. Suspended cells (i.e., oligodendrocyte lineage enriched) were decanted, filtered (100 µm pore nylon mesh), plated for 30 minutes to allow contaminating astrocytes to readhere and decanted again; adherent cells (i.e., astrocyte enriched) were

treated with 0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA) and recultured. Both were grown separately on coverslips for immunochemical analysis.

**Immunocytochemistry** A2B5 antibody (undiluted hybridoma supernatant), anti-GC antibody (diluted 1:40) and anti-GFAP antibody (1:300) were used to ascertain the percentage of oligodendrocyte lineage cells (A2B5-positive or GC-positive) and astrocytes (A2B5-negative, GFAP-positive) in the cultures. A2B5 antibody or anti-GC was added to unfixed cells and anti-GFAP after pre-treatment with 4% paraformaldehyde/0.05% Nonidet detergent. To double label, these steps were done in sequence. Primary antibodies were detected as follows: A2B5 with rhodamine-conjugated goat anti-mouse immunoglobulin (Ig) M (diluted 1:250); anti-GC with fluorescein-conjugated goat anti-mouse IgG (1:400); and anti-GFAP with either rhodamine or fluorescein-conjugated goat anti-rabbit IgG (1:400).

**MGMT Assays** MGMT activity in oligodendroglioma tissue was measured using a modified restriction endonuclease assay.<sup>15,16</sup> Values were expressed as percent activity relative to the MGMT positive,<sup>15</sup> BCNU resistant,<sup>16</sup> colon carcinoma cell line, HT29. The cell line SF767, grown as a xenograft in nude mice, was used as a second positive control. The negative control was an SF767 xenograft depleted of all MGMT activity by pretreatment of the tumor-bearing mouse with an alternate substrate for MGMT, O<sup>6</sup>-benzylguanine. Controls and HT29 samples were processed in the same manner as oligodendroglioma specimens and all samples were assayed simultaneously.

MGMT activity was measured in extracts prepared from oligodendrocyte lineage enriched and astrocyte enriched cultures using a DNA adduct removal assay described by Myrnes et al.<sup>17</sup> Cell cultures were washed in phosphate-buffered saline (PBS), suspended in a buffer containing 70 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM dithiothreitol, 1 mM EDTA and 5% glycerol, and sonicated. Extracts were incubated with calf thymus DNA containing O<sup>6</sup>-[<sup>3</sup>H]methylguanine for 30 minutes at 37°C. After the addition of 5% trichloroacetic acid (TCA) and heating to 80°C for 30 minutes, tritiated methyl proteins were recovered by filtration through glass fibre filters (Whatman GF/C). Filters were washed with 5% TCA/ethanol and radioactivity counted in a xylene based cocktail containing 0.5% diphenyl oxazole and 0.02% 1-4-bis-2(5-phenyl oxazolyl) benzene.

**Cytotoxicity Assay** The toxic effects of BCNU on oligodendrocyte lineage and astrocyte enriched cultures were compared using the MTT assay as modified for chemosensitivity testing by Cole.<sup>18</sup> BCNU was dissolved in absolute alcohol, diluted to 3.3 mg/ml with sterile water, and stored at -80°C. Subsequent dilutions were made with culture medium. Ninety-six well plates were seeded with oligodendrocyte lineage cells or astrocytes (10<sup>4</sup> cells/well) and incubated at 37°C. Two days later, BCNU (serial dilutions) was added for two hours then replaced with fresh medium. Four days later, 100 µl of medium was removed and 25 µl MTT solution (2 mg/ml in PBS) added and incubated for two hours at 37°C. To solubilize formazan crystals, 0.04M hydrochloric acid in isopropanol was added to each well and thoroughly mixed. Plates were kept at 37°C for one hour and cell viability quantified by measuring light absorbance (570 nm) with an automated reader. Dose response curves were normalized by expressing absorbance values relative to those for non-treated cells.

## RESULTS

Low levels of MGMT activity were found in five clinically aggressive, nitrosourea-responsive, human oligodendrogliomas. Activity levels were low relative to the MGMT positive, BCNU resistant, human colon carcinoma cell line, HT29. Levels in the tumor specimens ranged from 2.0 - 10.2% of that observed in HT29 cells (Table 1). To illustrate, patient 4, a 38-year-old woman with a multiply recurrent, previously irradiated, anaplastic oligodendroglioma had a complete response to PCV (Figure 1) and the tumor had low MGMT activity (i.e., 3.4%). She remains disease-free 30 months after starting chemotherapy treatment.

Oligodendrocyte lineage cells had low MGMT activity, approximately  $5 \times 10^3$  molecules per cell. MGMT levels in cells of the oligodendrocyte lineage were low relative to other rat cells,<sup>19</sup> and one-third that found in astrocytes ( $p < 0.02$ , Figure 2). A log-linear plot of cell viability versus BCNU concentration, shown in Figure 3, demonstrates that oligodendrocyte lin-

age cells were more sensitive to BCNU than astrocytes. The 50% inhibitory concentration ( $IC_{50}$ ) was 40  $\mu\text{g/ml}$  for oligodendrocyte lineage cells and 102  $\mu\text{g/ml}$  for astrocytes, a 2.5-fold difference. Oligodendrocyte lineage cultures in these studies were approximately 80% pure. They contained 40% A2B5-negative, GFAP-negative, GC-positive oligodendrocytes, 10% A2B5-positive, GFAP-negative, GC-negative oligodendrocyte progenitors, and 30% A2B5-positive, GFAP-positive, GC-negative cells [called type 2 astrocytes by some<sup>14</sup>]. They also contained 10 - 15% unidentified cells and 5 - 10% A2B5-negative, GFAP-positive, GC-negative astrocytes. Cultures enriched for astrocytes were at least 90% pure. Contaminants included small numbers of oligodendrocyte lineage and unidentified cells.

## DISCUSSION

One intriguing development in neuro-oncology in recent years has been the observation that aggressive oligodendrogliomas<sup>1,2</sup> and anaplastic mixed gliomas with an unequivocal oligodendroglial element<sup>3</sup> respond predictably to a combination chemotherapy regimen containing procarbazine, lomustine (CCNU) and vincristine, called PCV. Since oligodendrogliomas appear less vascular and less permeable than other gliomas,<sup>20</sup> superior drug delivery would seem an unlikely explanation for their chemosensitive nature. Presumably oligodendroglia cells are inherently susceptible to the cytotoxic effects of PCV, but why? The answer to this question is undoubtedly important as it may lead to better therapies for oligodendrogliomas and to new treatment strategies for anaplastic astrocytomas and glioblastomas.

The observation that oligodendrogliomas are preferentially induced in rats following transplacental exposure to ethylnitrosourea suggested to us that developing oligodendrocytes have difficulty repairing nitrosourea-induced DNA alkylation and that oligodendrogliomas might be chemosensitive if this biochemical

**Table 1:** MGMT Activity in Aggressive Oligodendrogliomas.

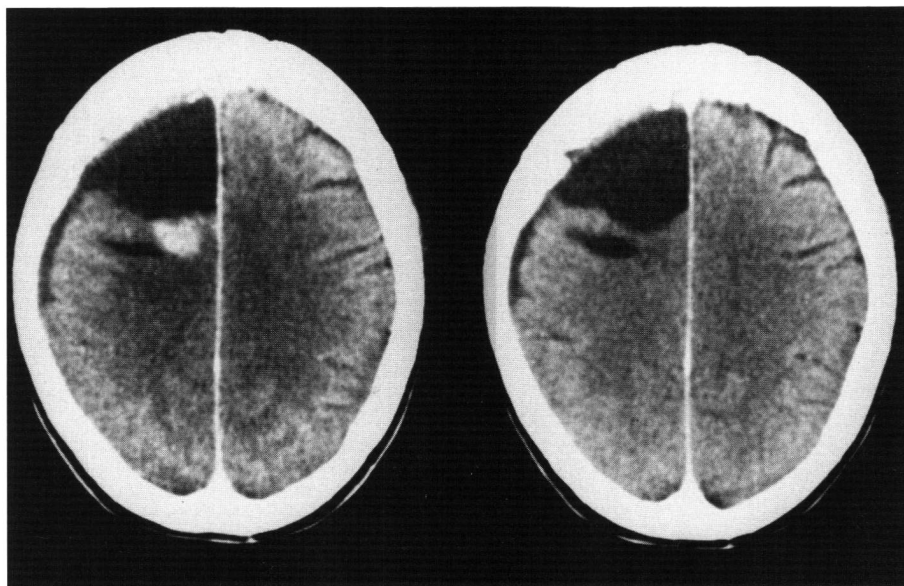
Case No.	Age/Sex	Prior Radiation	Chemotherapy Regimen <sup>a</sup>	Response Assessment <sup>b</sup>	% MGMT Activity <sup>c</sup>
1 <sup>d</sup>	49/F	No	PCV	CR	10.2
2	46/M	No	PCV	CR	2.0
3	41/F	No	PCV	PR	2.5
4	38/F	Yes	PCV	CR	3.4
5	35/F	Yes	PCV	CR	3.8

<sup>a</sup> PCV = procarbazine, CCNU (lomustine), vincristine

<sup>b</sup> CR = complete response; PR = partial response

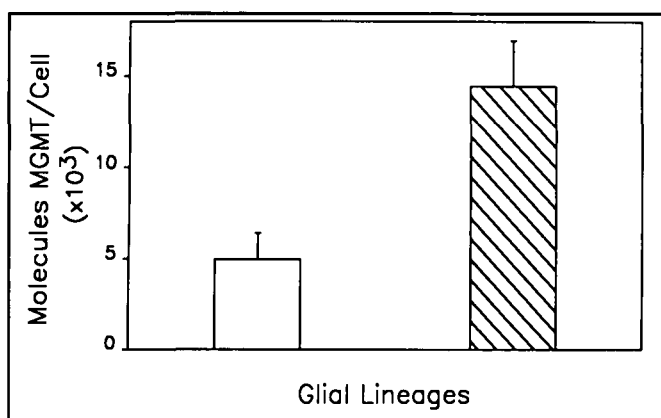
<sup>c</sup> Values are expressed as percent activity relative to the MGMT positive control, HT29 (HT29 level =  $2.28 \times 10^5$  molecules per cell<sup>14</sup>)

<sup>d</sup> enhancing, non-anaplastic oligodendroglioma

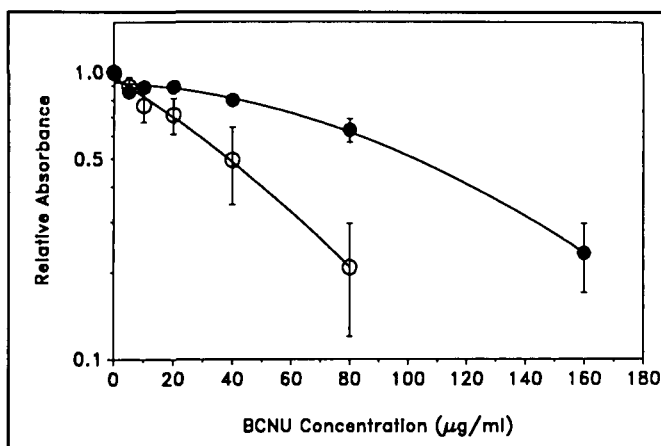


**Figure 1:** Computed tomographic scans before (left) and after (right) three cycles of PCV chemotherapy demonstrating disappearance of a contrast-enhancing, recurrent, anaplastic oligodendroglioma.





**Figure 2:** MGMT activity in glial cultures expressed as molecules/cell. Open box represents the average value for oligodendrocyte lineage enriched cultures ( $n = 5$ ) and the hatched box astrocyte enriched cultures ( $n = 6$ ). Bars depict the standard error of the mean ( $p < 0.02$ ).



**Figure 3:** Effect of BCNU concentration on glial cell viability. Closed circles represent average values for astrocyte enriched cultures ( $n = 18$ ) and open circles oligodendrocyte lineage enriched cultures ( $n = 6$ ). The absorbance of untreated cells was normalized to a value of 1.0. Bars depict the standard error of the mean.

vulnerability were retained in tumors of the oligodendrocyte lineage. Since MGMT repair of DNA alkylation confers resistance to nitrosoureas we reasoned further that rat oligodendrocyte lineage cells and human oligodendrogliomas might have low levels of MGMT activity, and they did. Allowing for the fact that rat tissues and cells have a several-fold lower level of MGMT than human tissues and cells,<sup>19</sup> both rat oligodendrocyte lineage cells and human oligodendrogliomas displayed low levels of MGMT activity relative to other rat and human tissues, respectively.

Neither we nor others have studied MGMT activity in human oligodendrocyte lineage cells, but several groups<sup>21-24</sup> have measured low levels in human oligodendrogliomas and we found low MGMT activity in PCV-responsive human oligodendrogliomas. Low levels of MGMT may have contributed to their chemosensitivity but only five tumors were studied. We also found that rat oligodendrocyte lineage cells had lower levels of MGMT and were more sensitive to BCNU than rat astrocytes. The significance of this finding in relation to the clinical observation that oligodendrogliomas in humans appear more sensitive

to PCV than anaplastic astrocytomas and glioblastomas, awaits further study. Wiestler et al.<sup>21</sup> and Forsina et al.<sup>22</sup> found lower levels of MGMT activity in oligodendrogliomas than astrocytomas but this has not been a universal finding.<sup>23,24</sup>

It is unlikely that the response of human cancer to chemotherapy is determined by a single biochemical process, such as MGMT. The observation that aggressive oligodendrogliomas respond not only to PCV and BCNU<sup>1</sup> but also to other alkylating agents, including diaziquone,<sup>1</sup> melphalan<sup>25</sup> and thiotepa,<sup>26</sup> raises the possibility that additional DNA repair mechanisms may be inefficient in oligodendroglial tumors. Studies of MGMT and other mechanisms of drug resistance in glial neoplasms, especially uncommon ones like oligodendrogliomas, are seriously limited by tissue availability. Moreover, contamination of samples by necrotic debris and non-tumor elements potentially complicate the interpretation of results. Our observation that human oligodendrogliomas and rat oligodendrocyte lineage cells have similarly low MGMT activity may be noteworthy in this regard. Perhaps normal rat glial cells may serve as a laboratory substitute for human gliomas if mechanisms of drug resistance in glial tumors reflect the biochemical properties of their cells of origin.

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