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MODULATION OF NITROSOUREA RESISTANCE IN HUMAN COLON CANCER BY O^6-METHYLGUANINE

STANTON L. GERSON,*† NATHAN A. BERGER, CHERYL ARCE, SHIRLEY J. PETZOLD and JAMES K. V. WILLSON

Departments of Medicine and Biochemistry, Ireland Cancer Center, Case Western Reserve University School of Medicine, and University Hospitals of Cleveland, Cleveland, OH 44106, U.S.A.

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Abstract—Human colon cancer is resistant to a variety of alkylating agents including the nitrosoureas. To specifically evaluate nitrosourea resistance, we studied the role of O^6-alkylguanine-DNA alkyltransferase (alkyltransferase) which is known to repair nitrosourea-induced cytotoxic DNA damage. Alkyltransferase activity varied over a similar wide range in 25 colon cancer biopsies and 14 colon cancer cell lines but the activity was not correlated with differentiation status, Dukes' classification or *in vitro* growth characteristics. 1,3-Bis-(2-chloroethyl)-1-nitrosourea (BCNU) resistance and alkyltransferase activity were highly correlated ($R^2 = 0.929$, $P < 0.001$) in 7 different colon cancer cell lines, suggesting that the alkyltransferase is an important component of nitrosourea resistance in colon cancer cells. In the BCNU-resistant, high alkyltransferase VACO 6 cell line, inactivation of the alkyltransferase by O^6-methylguanine caused a proportional decrease in the BCNU IC_{50}, consistent with that predicted by the regression line. Enzyme inactivation was also associated with a marked increase in DNA cross-link formation. Because alkyltransferase correlates with BCNU resistance in colon cancer, and resistance can be reversed by inactivating the protein, the alkyltransferase may have an important role in nitrosourea resistance in human colon cancer cells. These data provide the rationale for clinical trials in colon cancer with biochemical modulators of the alkyltransferase to increase the therapeutic response to nitrosoureas.

The majority of colon cancers are clinically resistant to conventional doses of chloroethylnitrosoureas but many are responsive to high-dose therapy [1, 2]. One explanation for this observed resistance pattern is that colon cancer cells may be able to efficiently repair drug-induced DNA damage. DNA damage induced by chloroethylnitrosoureas is initiated by the formation of various chloroethyl–DNA adducts of which O^6-chloroethylguanine appears to be important in cytotoxicity because it rearranges to form an N^1, O^6-ethanoguanine intermediate which is unstable and reacts with cytosine on the opposite strand, forming an interstrand DNA cross-link [3, 4]. These cross-links interfere with DNA replication and RNA transcription and can cause chromosomal aberrations and cell death [5, 6]. The formation of cross-links is closely associated with cytotoxicity [7].

Removal of non-bulky adducts at the O^6-guanine position is mediated by the DNA repair protein, O^6-alkylguanine-DNA alkyltransferase (alkyltransferase‡) (EC 2.1.1.63) [8, 9]. The alkyltransferase covalently transfers the alkyl group from the modified base adduct in DNA to a cysteine moiety within the active site of the protein resulting in irreversible inactivation of the alkyltransferase [10]. High levels of this protein correlate with nitrosourea resistance in leukemias [11], gliomas [12], rhabdomyosarcomas [13] and a number of other tumor cell lines [14, 15]. In contrast, cells with low levels of this repair protein are sensitive to nitrosoureas [11–16].

Whereas the alkyltransferase appears to confer nitrosourea resistance, it is possible to take advantage of the "suicide" reaction to sensitize cells to these agents. For instance, pretreatment with methylating agents to form O^6-methylguanine DNA adducts allows the alkyltransferase to be consumed through repair of the adducts [17, 18]. Subsequent exposure to chloroethylnitrosoureas yields DNA adducts that cannot be repaired by this mechanism resulting in increased sensitivity to the agents [17–19]. Previous studies have shown that pretreatment of resistant tumor cells with methylating agents or the modified bases O^6-methylguanine or O^6-benzylguanine [17–20] sensitizes the cells to chloroethylnitrosoureas and increases DNA damage as measured by sister chromatid exchanges and DNA cross-linking [19, 21, 22]. Dose modification factors of 2- to 12-fold have been observed with various cell lines which have high levels of alkyltransferase including the HT-29 colon cancer cell line [22], A549 lung cancer [22], HeLa cells [21], GR11 melanoma [23], XP mammary carcinoma [24], and the HL-60 promyelocytic cell line [25].

While these data provide a compelling rationale for the use of O^6-methylguanine to overcome nitrosourea resistance in the clinic, few studies have addressed the diversity of clinical tumors in terms of the heterogeneous nature of cell growth and of drug resistance mechanisms to define whether

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† Corresponding author: Dr. Stanton L. Gerson, Department of Medicine, University Hospitals of Cleveland, 2074 Abington Road, Cleveland, OH 44106. Tel. (216) 844-8532; FAX (216) 844-3000.

‡ Abbreviations: alkyltransferase, O^6-alkylguanine-DNA alkyltransferase (EC 2.1.1.63); and BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.

modulation of alkyltransferase will be a useful clinical modality to overcome tumor nitrosourea resistance. We have addressed this issue by studying a colon cancer cell line bank in which the morphology, growth and differentiation characteristics have been defined in detail [26, 27]. These cell lines may help define the importance of alkyltransferase in nitrosourea drug resistance in clinical colon cancer.

MATERIALS AND METHODS

Materials. 1,3-Bis-(2-chloroethyl)-1-nitrosourea (BCNU) and O^6-methylguanine were obtained from the Drug Synthesis and Chemistry Branch, Drug Therapeutics Program, National Cancer Institute. The BCNU was reconstituted fresh in 0.5 mL 100% ethanol at 22° and diluted to 10 mM in phosphate-buffered saline. [^{3}H]*N*-Methylnitrosourea (Amersham, Arlington Heights, IL) was used to synthesize ^{3}H-methylated DNA as described [16]. O^6-Methylguanine was synthesized from 6-chloroguanine and anhydrous sodium methoxide using a published method [28]. The preparation used contained 98.8 to 99.5% O^6-methylguanine by high pressure liquid chromatography analysis [11, 25]. Solutions of O^6-methylguanine were prepared fresh in warm 5% acetic acid, pH 5.2 [25].

Cell culture and treatments. The HCT 116 and Moser cell lines were provided by Dr. Michael Brattain, Bristol-Baylor Laboratory, Baylor College of Medicine [26]. The generation of the VACO cell lines has been described previously [27]. Briefly, cells were grown on plastic substrata in minimum essential medium with Earle's salts supplemented with 0.1 mM nonessential amino acids (Gibco), gentamicin (25 μg/mL), and 8% calf serum, and passage was limited to 20 cycles after which a new aliquot from an early passage was thawed. Drug inhibition of cell growth was analyzed by a cell regrowth assay as previously described [27]. Studies of the effect of BCNU alone were performed by adding BCNU in growth medium for 1 hr at 2×10^5 cells/mL, washing the cells in medium free of BCNU, and allowing the cells to grow for an additional 5 days in fresh medium before counting the number of viable cells in triplicate plates. To study the combined effect of O^6-methylguanine and BCNU, cells were preincubated in growth medium containing O^6-methylguanine, and BCNU was added 48 hr later. For alkaline elution studies, cells were pretreated with O^6-methylguanine for 24 hr before BCNU was added and then cultured in the same drug-containing medium for 6–24 hr before harvest for alkaline elution. This procedure decreased experimental variability due to loss of cells by washes and allowed for continued inhibitory effects of O^6-methylguanine. The potentiation of BCNU toxicity by O^6-methylguanine was determined by measuring the inhibitory concentration causing 50 or 90% [IC_{50} and IC_{90}] inhibition in cell growth. The paired *t*-test was used for a statistical test of significance.

Alkyltransferase assay: Sample preparation. Human colon cancer samples were dissected from primary colon cancer immediately after being removed from the patient. Samples were processed through the Case Western Reserve Cancer Center Tissue Procurement Core Facility. Care was taken to avoid necrotic areas and the absence of necrosis and the presence of tumor was verified histologically. The dissected samples were flash frozen over liquid N_2. Samples of adjacent normal mucosa were frozen after being stripped from the muscularis prior to freezing. Tissues were then thawed, dissociated using a Ten-Broch homogenizer in 3 volumes per weight cell extract buffer (70 mM HEPES pH 7.8, 0.1 mM EDTA, 5% glycerol and 1 mM dithiothreitol), at 4°, and processed identically to tissue culture cells. Tissue culture cells were washed twice in 50 mL PBS–1 mM EDTA at 4° and resuspended at 4×10^7 cells/mL in cell extract buffer; then cells or tissue homogenate was sonicated to complete cell disruption and centrifuged at 10,000 *g* for 2 min to remove cellular debris and the supernatant cytosol stored at −80° [25, 29].

Enzyme assay. [^{3}H]Methyl DNA was prepared as described [29] by reacting 4.0 mg calf thymus DNA in 0.01 M Ammediol buffer, pH 10, with 2 mCi [^{3}H]-*N*-methylnitrosourea at 35° for 15 min [29]. The major alkylation sites in this substrate are N^7-methylguanine (72%) and O^6-methylguanine (8.6%). The specific activity of the [^{3}H]methyl DNA substrate was 7.3 dpm of [^{3}H]O^6-methylguanine/fmol O^6-methylguanine. O^6-Alkylguanine-DNA alkyltransferase activity in cell extracts is measured as removal of the [^{3}H]methyl adduct from O^6[^{3}H]methylguanine in DNA alkylated with [^{3}H]*N*-methylnitrosourea as previously described [8, 29]. One femtomole of alkyltransferase activity is defined as the removal of 1 fmol of O^6-methylguanine and is expressed as fmol/μg DNA to compare different tissues and cells [30].

Alkaline elution assays. DNA cross-links and single-strand breaks were measured by the alkaline elution methods of Kohn *et al.* [31–33] under protein denaturing conditions [31–34]. L1210 cells labeled with [^{3}H]thymidine (6.7 Ci/mmol) for 24 hr were irradiated with 600 rads and collected on each filter to serve as internal elution standards. Treated and control colon cancer cells, prelabeled with 2-[^{14}C]-thymidine (54.0 mCi/mmol) for 72 hr during log growth at a concentration of 0.02 μCi/mL, were run directly and after irradiation with 600 rads to calculate DNA cross-links which are expressed in rad equivalents as previously described [31–34].

RESULTS

Alkyltransferase activity in human colon cancer. Alkyltransferase activity was assayed in 14 different human colon cancer cell lines, 25 colon cancer samples obtained from surgical biopsies and 15 samples of normal colon mucosa (Fig. 1). In this series of samples, the goal was to describe the distribution of alkyltransferase activity in colon cancer and normal colon mucosa. In many instances, tumor and normal mucosa samples were not paired from the same individual. Thus, it was not possible to directly compare the activity in an individual tumor with that in the adjacent normal mucosa. There was a wide range in alkyltransferase activity between samples in each group. This range is similar to the distribution in activity previously described in

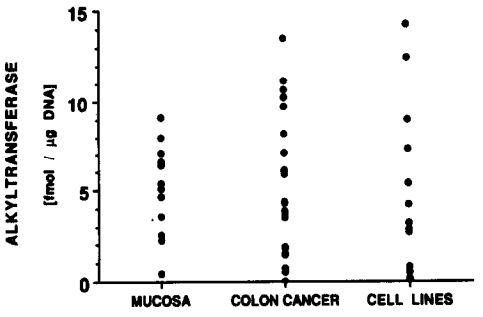

Fig. 1. Alkyltransferase activity in colon cancer. Normal colonic mucosa, colon cancer biopsies, and colon cancer cell lines were assayed for alkyltransferase activity. Each point represents an independent sample from either a separate patient or separate cell line and is the mean of 2–4 determinations.

normal and malignant cell lines and cells and tissues derived from patients and normal donors [11, 29, 30, 35–37]. This distribution appears to represent the spectrum of activity in different cell lines and tissues rather than experimental variation. Thus, the alkyltransferase activity among the 14 cell lines ranged from 0.2 to 16.4 fmol/μg DNA (mean ± SD, 5.4 ± 5.1 fmol/μg DNA) whereas the average variance in activity in the same cell lines (N = 7) assayed repeatedly over an 8-month period was only 17%. One sample of normal mucosa, three tumor biopsies and three cancer cell lines contained little or no detectable alkyltransferase activity, whereas others contained very high levels of activity. The lower levels of activity in the biopsy samples are not likely to be due to necrotic tumor because each sample was reviewed histologically and necrotic samples were excluded. Samples were obtained through the CWRU Cancer Center Tissue Procurement Facility which supplies these same samples for measurement of more labile substrates such as glutathione and mRNA. For this reason, inadequate sample procurement is rarely if ever observed. Thus, it appears that a small number of colon cancers (3/25) and colon cancer cell lines (3/14) are deficient in alkyltransferase.

The distribution of alkyltransferase activity in the colon cancer biopsies was similar to that observed in the colon cancer cell lines. Approximately one quarter of the colon cancer samples and cell lines contained higher alkyltransferase activity than observed in any of the samples of colonic mucosa. There was not a significant correlation between the alkyltransferase activity in the colon cancer biopsy samples and histologic grade ($P > 0.1$), Dukes' classification ($P > 0.1$) or with the alkyltransferase activity in the adjacent mucosa from the same individual ($P > 0.1$). Certain colon cancer biopsy samples and colon cancer cell lines contained as much alkyltransferase activity as the previously described HT29 cell line [22], which has 16.3 ± 1.8 fmol/μg DNA of alkyltransferase activity.

Correlation between alkyltransferase activity and BCNU resistance. Table 1 represents the features of the seven colon cancer cell lines utilized for further analysis and shows their wide distribution in doubling time. All are tumorigenic in nude mice. Table 1 also indicates the wide range in the BCNU IC_{50} and IC_{90}. As can be seen, there was no correlation between the BCNU responsiveness and doubling time of the cell lines. Figure 2 shows the correlation between alkyltransferase activity and the BCNU IC_{50}. A direct linear relationship was evident with a very high correlation coefficient, $R^2 = 0.929$, $P < 0.001$.

Modulation of alkyltransferase and BCNU resistance. To test the hypothesis that alkyltransferase activity was responsible for BCNU resistance in human colon cancer cell lines, O^6-methylguanine was used to inactivate the alkyltransferase and then to modulate BCNU resistance. Figure 3 shows the concentration-dependent inactivation of alkyltransferase in the VACO 6 cell line following a 48-hr exposure to increasing concentrations of O^6-methylguanine. Alkyltransferase activity was decreased by 86% during exposure to 0.2 mM O^6-methylguanine and by 95% with 0.5 mM O^6-methylguanine.

Figure 4A shows that treatment of the highly sensitive VACO 8 cell line with O^6-methylguanine resulted in no alterations of BCNU cytotoxicity. Figure 4B shows the effect of preincubating the VACO 6 cell line in 0.2 mM O^6-methylguanine for 48 hr prior to exposure to increasing concentrations of BCNU. Cells exposed to O^6-methylguanine continued to be grown in medium containing 0.2 mM O^6-methylguanine for the duration of the experiment to prevent regeneration of alkyltransferase activity. Inactivation of the alkyltransferase in VACO 6 cells resulted in increased BCNU cytotoxicity with a reduction in the IC_{50} of approximately 2.2-fold and in the IC_{90} of 2.4-fold.

Modulation of BCNU-induced cross-links. Figure 5 shows the concentration-dependent effects of BCNU-induced DNA cross-links. BCNU-sensitive VACO 8 cells show a clear concentration-dependent increase in DNA cross-links whereas BCNU-resistant VACO 6 cells failed to show any significant cross-link formation. The DNA-cross-link level after 24 hr of incubation was similar in each cell line to that observed after 6 hr. The apparent negative values for cross-links in the VACO 6 cells have been observed previously with nitrosoureas [31] and are associated with the occurrence of drug-induced DNA strand breaks. Table 2 shows the change in DNA cross-links that occurred with the combination of O^6-methylguanine and BCNU. In VACO 6 cells, O^6-methylguanine pretreatment produced a marked increase in DNA cross-links. In VACO 8 cells, the combination produced essentially no increase in DNA cross-links. This is similar to the effect of O^6-methylguanine on BCNU-induced cytotoxicity in the two cell lines (Fig. 4).

DISCUSSION

A high degree of correlation has been found between BCNU resistance and alkyltransferase activity in a large number of human colon cancer cell lines. These data provide one explanation for

Table 1. *In vitro* cytotoxicity of BCNU against a panel of human colorectal cell lines

Cell line	BCNU (μg/mL) IC_{50}	IC_{90}	Alkytransferase* (fmol/μg/ DNA)	Doubling time (hr)
VACO 8	1.5	3.5	0.05 ± 0.01	46
VACO 5	4.5	12	0.1 ± 0.1	29
Moser	6.0	11	0.8 ± 0.6	30
HCT 116	7.5	15	5.4 ± 1.3	20
VACO 9P	13	42	9.0 ± 1.8	110
VACO 6	19	>50	12.4 ± 0.9	26
VACO 10M	11	>50	7.3 ± 0.9	36

* Values are means ± SEM; each cell line was assayed in duplicate 3–7 times.

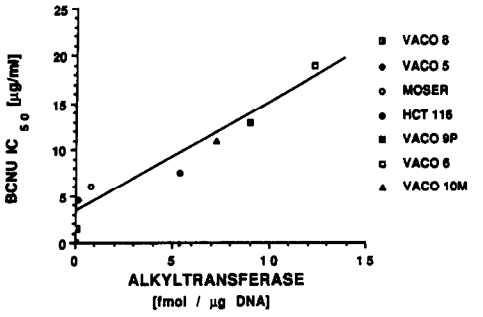

Fig. 2. Correlation between alkyltransferase activity and BCNU cytotoxicity. The mean alkyltransferase activity and mean IC_{50} were plotted for each of 7 colon cancer lines in the cell bank. The linear regression line shown satisfies the equation $y = 3.1 + 1.17x$. The regression coefficient was $R^2 = 0.929$, $P < 0.001$.

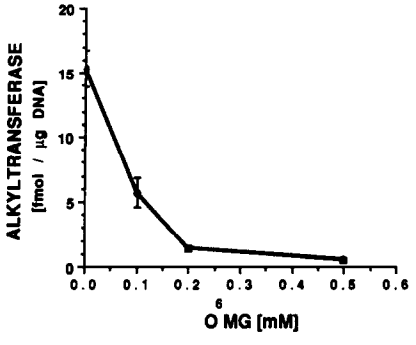

Fig. 3. Inactivation of alkyltransferase in VACO 6 by O^6-methylguanine: VACO 6 cells were prepared as described in Materials and Methods and incubated in increasing concentrations of O^6-methylguanine (OMG) for 48 hr prior to being assayed for residual alkyltransferase activity.

the observed clinical resistance of human colon cancer to nitrosoureas and provide a rationale for attempting to overcome nitrosourea resistance through inactivation of alkyltransferase activity. The diversity of morphologic and growth characteristics of the human colon cancer cell lines studied and the similar range of alkyltransferase activity in the human colon cancer samples and the cell bank suggest that this bank may be representative of clinical colon cancer and that the drug resistance patterns and their modulation may be similar as well. As such, this bank provides an important model with which to verify drug resistance mechanisms in human colon cancer.

Previous studies suggest that very low levels of alkyltransferase activity observed in tumor cell lines may be an artifact of tissue culture and not seen in primary human tumors [16, 38]. However, our study of human colon cancer showed that the proportion of fresh tumors which contained very low levels of alkyltransferase was similar to that observed in cell lines derived from these tumors. Redmond *et al.* [39] recently reported alkyltransferase measurements in 23 samples of human colon cancer and adjacent normal mucosa and found a somewhat smaller range in activity than we observed. Both studies found that about 20–25% of human colon tumors had higher alkyltransferase activity than any sample of normal mucosa. While the alkyltransferase activity in human cell explants does not always correlate directly with the alkyltransferase activity from the primary cells or tissue from which it is derived [40], suggesting that there may be both normal and tumor cell heterogeneity in expression of alkyltransferase activity, our data suggest that the distribution of alkyltransferase activity in the colon cancer cell line bank is representative of the pattern of activity observed in human colon cancer tumors.

A correlation between alkyltransferase activity and nitrosourea resistance has been seen in a variety of tumor cell lines [41, 42]. However, our study is the first to document a linear relationship between alkyltransferase and BCNU cytotoxicity in a diverse set of colon cancer cell lines. Furthermore, our data suggest that inactivation of the alkyltransferase by O^6-methylguanine reduces the IC_{50} to the extent predicted by the regression line (Fig. 2). This finding is remarkable given that there are a number of potential etiologies of BCNU resistance in clinical tumors including the contributions of drug uptake, glutathione conjugation [43], bioavailability [44], and polyamine metabolism [45]. Elevated P-glycoprotein, glutathione, glutathione-*S*-transferase and glutathione peroxidase have been observed in

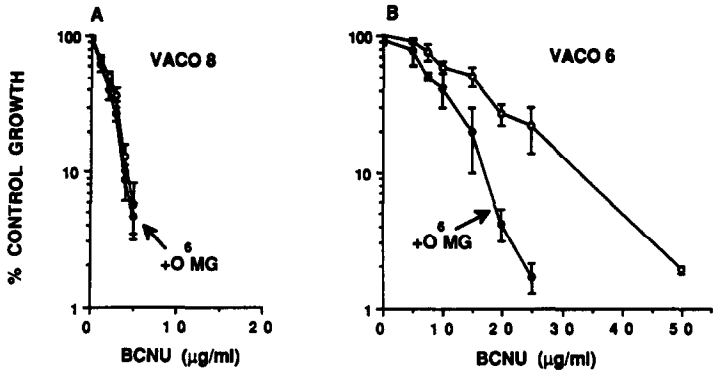

Fig. 4. Potentiation of BCNU cytotoxicity by O^6-methylguanine. VACO 8 (A) or VACO 6 (B) cells were preincubated in 0.2 mM O^6-methylguanine for 48 hr and then exposed to increasing concentrations of BCNU in the cell regrowth assay, as described in Materials and Methods. At harvest, cell viability was over 95%. Each point is the mean ± SD of three independent experiments.

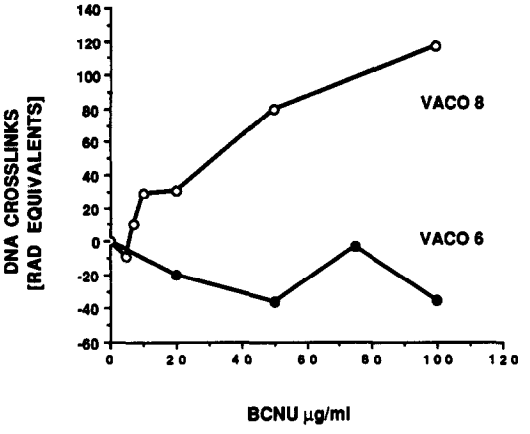

Fig. 5. Concentration-dependent BCNU-induced DNA cross-links. DNA cross-links were produced by incubating VACO 6 and VACO 8 cell lines with increasing concentrations of BCNU for 6 hr at 37°. Each cell line was prelabeled with [^{14}C]thymidine and examined in duplicate at each drug concentration using [^{3}H]thymidine-prelabeled L1210 cells to monitor elution kinetics as described in Materials and Methods. Cell viability at the time of harvest was over 95%.

Table 2. Increase in DNA cross-links (rad equiv.) induced by O^6-methylguanine over that of BCNU alone

	Induced DNA cross-links*	
	duration of treatment	
Cell lines	6 hr	24 hr
VACO 6	139	163
VACO 8	26	8

* VACO 6 and VACO 8 cells lines were pretreated with 0.4 mM O^6-methylguanine for 24 hr and then treated with 100 μg/mL BCNU and 0.4 mM O^6-methylguanine for an additional 6 or 24 hr. Control cells were treated either without any drugs, with O^6-methylguanine or with BCNU alone. DNA cross-links were measured by alkaline elution at the end of the 6- or 24-hr treatment with BCNU and O^6-methylguanine as described in Fig. 5 and Materials and Methods. DNA cross-links were converted to rad equivalents; increases in BCNU-induced DNA cross-links due to treatment with O^6-methylguanine were determined by subtracting the cross-links measured in cells treated with BCNU alone from those measured in cells treated with the combination of O^6-methylguanine and BCNU.

some human colon cancer tumors [39], suggesting that these resistance mechanisms may play an important role in BCNU resistance in some tumors. These mechanisms may be important *in vitro* as well because the regression line between the BCNU IC_{50} and alkyltransferase activity for the colon cancer cell lines failed to go through the origin, suggesting that one or another of the above mechanisms is a second line of resistance to BCNU in human colon cancer. Because our results identify the alkyltransferase as a dominant mechanism of BCNU resistance in colon cancer cell lines, they raise the possibility that even a low level of alkyltransferase may be sufficient to prevent human colon cancer from responding to nitrosoureas in the clinical setting. Alternatively, human colon cancers may be markedly heterogenous in their expression of a variety of drug resistance mechanisms and the alkyltransferase may be part of a complex system of protection.

Nonetheless, our studies document that modulation of alkyltransferase resistance can impact on nitrosourea resistance in human colon cancer, at least *in vitro*. To be successful, any biochemical modulation program must improve the "therapeutic index" of the combination over that available with the chemotherapeutic agent alone. This may be a problem with the alkyltransferase because direct inhibitors inactivate the protein in most tissues and are unlikely to provide any tissue-specific selectivity. In other studies, we have used xenografts of the colon cancer cell lines described in this study to measure the modulation of alkyltransferase *in vivo*

and to determine the degree to which BCNU resistance can be reversed in a pre-clinical model using O^6-benzylguanine [46], based on the work of Dolan *et al.* [20]. These models will also define whether biochemical modulation of alkyltransferase *in vivo* allows tumor regression using tumors which are resistant to BCNU alone. Similar studies are underway in clinical trials using streptozotocin to inactivate the alkyltransferase, followed by BCNU in an effort to potentiate the therapeutic response. Both xenograft and clinical trials will determine whether this approach will yield a better therapeutic index for the use of BCNU in the treatment of solid tumors such as colon cancer.

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