1994

The interaction of O6-benzylguanine and streptozotocin in the inactivation of O6-methylguanine DNA methyltransferase and the reversal of 1,3 bis (2-chloroethyl)-1-nitrosourea resistance

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THE INTERACTION OF O^6-BENZYLGUANINE AND STREPTOZOTOCIN IN THE INACTIVATION OF O^6-METHYLGUANINE DNA METHYLTRANSFERASE AND THE REVERSAL OF 1,3 BIS(2-CHLOROETHYL)-1-NITROSOUREA RESISTANCE

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

BY

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CHICAGO, ILLINOIS

MAY 1994

DEDICATION

To Uma, Ramya, Gopi, and Prakash

In the game of science-or life-the highest goal isn't to win it's to win at something really difficult... it's to go somewhere beyond your capability and come out on top.

James D. Watson
Science,(261) p.1812
1993

ACKNOWLEDGEMENTS

I have been fortunate to have had a number of profoundly positive influences which have made this work possible. I thank my parents, Munaswamy and Pushpa (my life long friends who have offered altruistic and unconditional love and support), for instilling the importance of family and education. To Chuck Layton, my oldest friend and horse trainer, I offer my sincerest thanks for incessantly reminding me to always "make a plan" and "keep your ride going forward".

I also greatfully acknowledge my labmates, Greggory Herbert, Leah Santiago, Dr. Roger Kroes, Dr. Qing Dong, Laura Dawson, and Emanuel Selg for always providing helpful hands and empathetic ears. I thank Dr. Leonard Erickson for teaching the meaning of the phrase "attention to detail." I owe Dr. M. Eileen Dolan a tremendous debt of gratitude for a fruitful collaboration. Finally, I thank the rest of my dissertation committee members Drs. Israel Hanin, Russell Pieper, and Thomas Ellis for their guidance, support and insightful discussions throughout my tenure in graduate school.

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LIST OF ABBREVIATIONS

ACNU	1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea
ANOVA	analysis of variance
BCNU	1,3-bis(2-chloroethyl)nitrosourea
BG	O^6-benzylguanine
cDNA	complementary DNA
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CENUs	chloroethylnitrosoureas
CNU	1-(2-chloroethyl)-1-nitrosourea
CPM	counts per minute
dBG	O^6-benzyl-2'-deoxyguanosine
g	grams
X g	acceleration due to gravity
GSH	glutathione
GST	glutathione-S-transferase
hr	hour
HEPES	N-[2-hydroxyethyl] piperazine-N'-2-ethane sulfonic acid
i.p.	intraperitoneal
IC_{50}	concentration of a compound producing 50% inhibitory effect
meCCNU	1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea

Mer	methylation repair
MG	O^6-methylguanine
MGMT	O^6-methylguanine DNA methyltransferase
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	1-methyl-1-nitrosourea
°C	degrees Celsius
PCI	phenol:chloroform:isoamyl alcohol
pmol	picomole
SD	standard deviation
STZ	streptozotocin

CHAPTER I

INTRODUCTION

Although contemporary medicine has celebrated remarkable success in the treatment of symptoms and etiology of microbial infections, the incidence of progressively debilitating diseases such as cardiovascular, neoplastic and neurological diseases has increased unabated. Second only to heart disease in incidence, cancer is a disease characterized by germ line mutations and progressive accumulation of somatic mutations in genes regulating cellular growth and differentiation. Of over forty chemotherapeutic agents approved for clinical use, no single, polypharmaceutical, or multi-modality therapies have served as the "mithridieum", or the magic bullet, for the eradication of neoplastic disease in humans.

The oncologist has used chemotherapy as a front line therapy, or in an adjuvant setting with radiation therapy, hypothermia, and surgery in combating neoplastic disease. Curative regimens for the relatively rare adult neoplastic transformations of testicular and hematopoietic cells have been developed. However, more common forms of neoplasms such as colon, lung, and breast

cancer have been refractory to chemotherapeutics which target neoplastic DNA and intermediary metabolism.

Drug resistance can be defined as the ability of the tumor cell to survive exposure to cytostatic and cytocidal agents when administered at the maximum dose tolerated by normal cells. This phenomenon is an interactive consequence of a number of host and tumor cell factors. Host factors include drug absorption, distribution, metabolism, and excretion which can decrease the bioavailability of agents to the tumor site. Tumor cells possess a number of biochemical mechanisms of drug resistance that decrease the intracellular bioavailability of chemotherapeutic agents and facilitate the repair of macromolecular damage inflicted by these drugs. The ultimate obstacle to therapeutic success is the ability of the tumor cell to survive relatively lethal concentrations of chemotherapeutic agents (O'Brien and Cordon-Cardo, 1991). Therefore, the identification and selective inactivation of drug resistance mechanisms could increase the therapeutic index of an anti-neoplastic agent.

A class of anti-neoplastic agents, the chloroethylnitrosoureas (CENUs), has been employed in the treatment of primary brain tumors, malignant melanoma, Hodgkin's and non-Hodgkin's lymphomas, and multiple myeloma, with encouraging results (Young, et al., 1971;

Walker, 1973; Wilson, et al., 1976, Mortel, 1978). However, the CENUs produce anti-tumor responses in fewer than 20% of patients with non-hematological malignancies, suggesting that the majority of human malignancies are inherently resistant to the cytotoxic effects of these agents (Carter, et al., 1976). Evidence suggests this limited effectiveness of CENUs is due to the presence of the DNA repair protein, O^6-methylguanine DNA methyltransferase (MGMT) in tumor cells (Myrnes et al., 1984,; Chen, et al., 1992; Citron, et al.1991). This protein prevents the formation of the cytotoxic lesion induced by CENU, the DNA interstrand cross-link (Erickson, et al., 1980a; Yarosh, et al., 1983).

This dissertation describes pre-clinical studies aimed at developing a novel chemotherapeutic regimen that targets the extended depletion of MGMT in order to increase the therapeutic effectiveness of one of the CENUs, 1,3 bis(2-chloroethyl)-1-nitrosourea (BCNU). This regimen consists of the sequential administration of two MGMT depleting agents, O^6-benzylguanine (BG) and streptozotocin (STZ), prior to BCNU treatment. Considerable evidence suggests that pretreatment of resistant cells with MGMT depleting agents prior to CENU exposure increases DNA cross-link formation and cytotoxicity in resistant cells (Zlotogorski and Erickson, 1983, 1984; Dolan et al., 1988; Dolan et al.,

1990a,b). The observation that the DNA interstrand cross-link precursor O^6, N^1 ethanoguanine is stable in CENU treated DNA for greater than 8 hr following drug removal suggests that the potential for cross-link formation exists for extended periods (Brent, 1985b). DNA interstrand cross-links produced by CENUs continue to form 6-12 hr after drug removal in MGMT deficient cells (Erickson et al., 1980a). These data suggest that extended depletion of MGMT throughout the time-course of cross-link formation could maximize CENU cross-linking and cytotoxicity. If functional MGMT recovers during the time-frame of cross-link formation in resistant cells, then the enhancement of CENU cytotoxicity by MGMT depleting agents would be sub-maximal. The importance of recovery of MGMT activity following MGMT depleting agents in determining the potentiation of CENU cytotoxicity has largely been neglected. Therefore, experiments described within this dissertation test the following hypothesis; depletion of MGMT prior to, and for an extended period following BCNU administration maximizes BCNU anti-tumor activity in resistant cells. In testing this hypothesis, the combination of O^6-benzylguanine (BG) and streptozotocin (STZ) was used in an effort to produce a more extended depletion of MGMT, and a greater enhancement of BCNU cytotoxicity than either STZ or BG alone. The enhancement of BCNU cytotoxicity was measured

under conditions of extended depletion of MGMT for 24 hr with the combination of BG+STZ, partial or complete recovery of MGMT by 24 hr with BG or STZ as single agents. The combination of BG+STZ produced a more prolonged depletion of MGMT and potentiated BCNU cytotoxicity to a greater extent than either BG or STZ alone. However, the combination of BG+STZ+BCNU provided no greater anti-tumor activity than BG+BCNU in vivo. The combination of BG+BCNU produced greater anti-tumor activity than STZ+BCNU, and equivalent anti-tumor activity to that of the three-drug combination. These data demonstrate that extended depletion of MGMT following BCNU treatment is required to maximize BCNU cytotoxicity. Additionally, the sequential use of BG, STZ and BCNU probably results in a greater number of STZ lesions which are responsible for the continued depletion of MGMT and the greater enhancement of BCNU cytotoxicity in vitro.

Collectively, these data suggest that clinical regimens targeting the inactivation of MGMT and the reversal of CENU resistance should be optimized such that MGMT is depleted for extended periods following BCNU administration. Although BG can efficiently deplete MGMT for prolonged periods in vitro and in vivo, it may not be possible to achieve ample concentrations of BG to inactivate MGMT in tumors with high levels of the protein

for a prolonged period. A possible means of reaching this objective is by the combined use of the two MGMT depleting agents, BG and STZ.

CHAPTER II

REVIEW OF RELATED LITERATURE

Upon organization of the National Cancer Institute's Chemotherapy Program in 1955, several transplantable mouse tumors were selected for the evaluation of potential anti-neoplastics because clinically useful chemotherapeutic agents were effective against one or more of these tumors (Gellhorn and Hirschberg, 1955). Among these, systemic leukemia L1210 cells responds to the greatest number of novel compounds of potential clinical interest.

The 2-chloroethyl derivatives of the DNA methylating agents 1-methyl-3-nitro-nitrosoguanidine (MNNG) and 1-methyl-1-nitrosourea (MNU) showed greater anti-tumor activity than the parent compounds in the L1210 leukemia model (Skinner et al., 1960, Johnston et al., 1963). Shortly thereafter, Skipper et al. (1964) at the Southern Research Institute demonstrated the curative ability of BCNU against L1210 cells. 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (meCCNU) were subsequently synthesized and showed similar anti-tumor activity against L1210 leukemia and Lewis lung carcinoma

implants (Schabel, 1967, Mayo et al., 1972). The anti-tumor activities of the 1,3 di-substituted nitrosoureas against a wide variety of rodent neoplasms has been compiled by Schabel (1976). This remarkable anti-tumor activity produced by CENUs in mice led to the clinical use of BCNU (Carmustine), CCNU (Lomustine), meCCNU (Semustine), chlorozotocin, clomesone, and 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU).

Mechanism of Action of Chloroethylnitrosoureas

The macromolecular damage caused by the CENUs includes alkylation of nucleic acids and carbamoylation of lipids and proteins. However, their antitumor activity appears to be the consequence of DNA alkylation. Among the plethora of base modifications, the di-adduct DNA interstrand cross-link has been demonstrated as the prominent cytotoxic lesion (Ewig and Kohn, 1977; Erickson et al, 1980 a,b; Kohn et al, 1981).

At physiologic pH, CENU decompose into an alkylating moiety (a chloroethyl diazo hydroxide which further decomposed to yield a chloroethyl carbonium ion) and a carbamoylating moiety (chloroethyl isocyanate) (Montgomery, et al., 1967). The formation of the DNA interstrand cross-link occurs in a two-step reaction. Cross-linking is thought to involve primary

chloroethylation of a nucleophilic site on one strand of DNA, with nucleophilic displacement of Cl^-, and subsequent reaction with the opposite strand of DNA, resulting in a covalent alkyl bridge between the two strands of DNA (Kohn, 1977). Initially, the chloroethyl carbonium ion reacts with the O^6 position of guanine within DNA and produces O^6-chloroethylguanine (Tong et al., 1981). This monoadduct undergoes a slow intramolecular rearrangement to form an O^6, N^1-ethanoguanine. This cyclized intermediate reacts with its complementary base cytosine to form N^1-guanine, N^3-cytosine DNA interstrand cross-link (Tong et al., 1982). The initial alkylation and cyclization requires a low activation energy and is complete within minutes, whereas the later reaction necessitates a higher activation energy and can take up to 6-10 hr at 37°C for completion (Lown et al., 1978). This time-course of cross-link formation appears to be similar in isolated DNA (Kohn, 1977; Lown et al., 1978) and DNA repair deficient cells (Erickson et al., 1980a). The formation of DNA interstrand cross-links correlates with inhibition of DNA replication and RNA transcription, induction of G_2 arrest and cytotoxicity (Gralla et al., 1987; Pieper et al., 1989; Jiang et al., 1989). The importance of DNA cross-linking is further evidenced by the sensitization of CENU resistant cells with drug resistance modulators. Depletion of the DNA repair

protein MGMT by STZ or BG results in an increased number of cross-links and an enhancement of CENU cytotoxicity (Futscher et al, 1989; Mitchell et al, 1992). These studies will be described further below.

Alkylation of DNA is currently thought to be of greater importance in mediating the anti-tumor activity of CENUs than carbamoylation damage. Although the rank order of carbamoylating activity of a number of nitrosoureas does not correlate with anti-tumor activity (Heal et al, 1979; Wheeler et al, 1974), this activity may have clinical importance. The effects of carbamoylation on cellular metabolism have been extensively reviewed by Lea (1987).

The primary clinical implications of macromolecular carbamoylation damage resides in the CENU-mediated inhibition of DNA repair and in the delayed myelosuppression caused by CENU (Sarabin et al., 1984; Ali-Osman, 1985). The chloroethyl isocyanate carbamoylates the epsilon amino groups of lysine or arginine residues in various cellular proteins (Holtham and Schutz, 1949). Carbamoylation alters protein solubility and thereby limits protein function (Holtham and Schutz, 1949).

Carbamoylation and the Inhibition of DNA Repair

Using organic isocyanate derivatives of CENU, Kann et al. (1974) demonstrated a marked inhibition in the repair of X-irradiation-induced DNA single strand breaks. A subsequent study by Fornace et al. (1978) identified the step most sensitive to carbamoylation damage as the re-ligation of ultraviolet radiation-induced single strand breaks. The 2-chloroethyl isocyanate did not impair the production of strand breaks and repair polymerase activity, but inhibited DNA ligase activity. These observations suggest that the impairment of DNA ligase activity is indicative of the inhibition of an excision repair type mechanism which may contribute to the cytotoxicity arising from CENU alkylation damage. However, numerous studies suggest that carbamoylation damage can inhibit DNA repair but can not substantially contribute to the therapeutic effectiveness of CENU. Clinically relevant doses of 2-chloroethyl isocyanate (i.e. those approximated to be produced by 100μM BCNU and CCNU) do not inhibit repair of gamma-irradiation-induced strand breaks and are non-toxic to L1210 or HeLa cells (Hilton et al., 1978). This contention is further supported by the comparative effects on DNA repair and cytotoxicity of two D-glucopyranosyl derivatives of MNU with similar alkylating properties and divergent carbamoylating activity. The inhibition of DNA repair by

the carbamoylating agent 3-beta-D-glucopyranosyl-1-methyl-1-nitrosourea (GMNU) does not produce any greater cytotoxicity than its non-carbamoylating congener STZ (Heal, et al, 1979). This inhibition of DNA repair could have clinical relevance in radioresistance and may explain the observed therapeutic synergism by the combined administration of two CENUs (Kann et al, 1980; Skipper, 1965). The induction of DNA strand breaks appears to be a primary mechanism of cell killing by X-irradiation. Therefore, potent carbamoylating agents like 1,3-bis (trans-4-hydorxycyclohexyl)-1-nitrosourea (BCyNU) could be used to enhance the anti-tumor activity of ionizing radiation (Kann et al., 1980). Similarly, the synergism between combined administration of two CENUs could also be attributed to the inhibition of DNA repair. The direct interaction of CENUs and partially purified MGMT results in the inactivation of the protein (Brent, 1985a), presumably via carbamoylation. Furthermore, excision repair has been implicated in BCNU resistance (Swinnen, et al., 1991). Therefore, inactivation of one or both of these repair systems could be the basis of the synergistic interaction of CENUs observed when they were co-administered against rodent tumors.

Carbamoylation and CENU Induced Myelosuppression

Delayed myelosuppression is the dose limiting toxicity of CENUs (Carter et al., 1972; Schein et al., 1975; Montgomery, 1976; Johnston, et al., 1986). The involvement of carbamoylation damage in the etiology of myelosuppression is ambiguous. The relative lack of bone marrow toxicity of chlorozotocin (a CENU devoid of in vitro carbamoylating activity and potent DNA cross-linking ability) suggests carbamoylation positively relates to myelosuppression (Anderson et al., 1975; Panasci et al., 1979). However, other evidence suggest that carbamoylation may not be responsible for myelosuppression. The relative in vitro carbamoylating activity of several CENUs and methylnitrosoureas does not correlate with bone marrow toxicity in mice (Panasci et al., 1977; Brubaker et al., 1987). Only a modest relationship exists between the in vitro carbamoylation activity and myelosuppression (Heal et al., 1979). ACNU and 1-(2-chloroethyl)-3-(1-deoxycyclo-inositol)-1-nitrosourea, which has little in vitro carbamoylating activity, produces comparable bone marrow toxicity to the potent carbamoylating agent CCNU. Additionally, another potent carbamoylating agent 1-(2-chloroethyl)-3-(beta-D-glucopyranosyl)-1-nitrosourea (GANU) is not myelosuppressive. The significance of these data is difficult to evaluate in light of the observations made

by Gibson and Hickman (1982) that the in vitro carbamoylating activity may not be predictive of in vivo carbamoylation. Experiments comparing BCNU, chlorozotocin, GANU, and BCyNU-induced myelosuppression suggest that myelosuppression may be associated more with the qualitative types of carbamoylation damage than the amount of damage (Ali-Osman et al., 1985).

The Relative Importance of Alkylation and Carbamoylation Damage in CENU Cytotoxicity

A reductionist consideration of nitrosourea mechanism of action in terms of alkylation versus carbamoylation damage is "obviously simplistic" (Lea, 1987). The type of carbamoylating moiety within a CENU determines its lipid solubility and influences a number of pharmacokinetic parameters (Hansch et al., 1972; Levin and Kabra, 1974). Once the drug localizes to the tumor, alkylation damage could be of primary importance in cytotoxicity (Lea, 1987). As previously stated, the rank order of carbamoylating activity of a number nitrosoureas does not correlate with the anti-tumor activity (Heal, et al., 1979; Wheeler, et al., 1974). Chlorozotocin, a CENU with high cross-linking ability and minimal carbamoylating activity, produces marginal anti-tumor activity compared to congeners with both carbamoylating and chloroethylating activity (Gibson and Hickman, 1982).

Another potent carbamoylating agent, BCyNU devoid of alkylating activity does not produce anti-tumor activity (Kann et al., 1980). The compound cis-2-OH-CCNU which has lower carbamoylating activity than its parent compound CCNU retains full anti-tumor activity (Wheeler, 1977). Furthermore, a convincing body of data correlating CENU anti-tumor activity and DNA cross-linking in tumor cell lines and xenografts suggests that the primary mode of cell killing is due to alkylation of DNA (Kohn et al., 1981; Thomas et al., 1978).

Mechanisms of CENU Resistance

Tumor cells become resistant by: 1) decreasing cellular drug uptake and/or increasing drug efflux (Waud., 1987; Vendrik et al., 1992); 2) increasing intracellular detoxification of xenobiotics (Litterst, et al., 1985; Waxman, 1990; Teicher and Frei, 1991); 3) over-producing target proteins (Nunberg et al., 1978); 4) decreasing levels of bioactivating enzymes (Dulhanty, et al., 1989; Hoban et al., 1990); 5) increasing repair of macromolecular damage (Barrows, et al., 1987; Batist, et al., 1989; Bedford et al., 1988; Bodell, et al., 1988). The plasma membrane protein P-glycoprotein facilitates active efflux of a number of structurally unrelated chemotherapeutic agents (Vendrik, et al., 1992). Intracellular thiols such as glutathione and

metallothionine act as nucleophilic sinks for a number of DNA damaging agents (Teicher and Frei, 1991). Anti-metabolite drug resistance is imparted by the over production of target proteins. A well characterized example of this type of mechanism is the amplification and over- expression of the dihydrofolate reductase gene in mediating methotrexate resistance (Nunberg et al., 1978). The decreased bioactivation of the pro-drug mitomycin C appears to involve compromised microsomal function (Hoban et al., 1990). The mechanism of primary importance to this dissertation is the increased cellular capacity to repair macromolecular damage. The mediation of CENU resistance by the DNA repair protein MGMT and modulation of such resistance will be extensively discussed. Furthermore, several secondary mechanisms implicated in CENU resistance will also be discussed below.

MGMT and CENU Resistance

The CENU-induced DNA interstrand cross-link is formed via an O^6-chloroethylguanine with subsequent cyclization yielding O^6, N^1-ethanoguanine (Tong, et al., 1981, 1982). This monoadduct interacts with the complementary base cytosine and results in a guanine-cytosine DNA interstrand cross-link. Completion of cross-link formation occurs in approximately 6-10 hr (Kohn, 1977;

Erickson et al., 1980a; Brent, 1985). This protracted time course for cross-link formation allows the repair of cross-link precursors by the 22 kd MGMT protein and prevents the accumulation of the lethal lesions (Brent, 1985a).

Tumor cell lines that prevent the formation of CENU-induced DNA interstrand cross-links are resistant to CENU cytotoxicity (Erickson et al., 1980 a,b). This resistance phenotype correlates well with the ability of a cell line to reactivate MNNG-damaged adenovirus. The phenotypic proficiency or deficiency in the reactivation of damaged adenovirus is referred to as **ME**thylation **R**epair positive (MER^+) or **ME**thylation **R**epair negative (MER^-), respectively (Day et al., 1980a). The MER phenotype (Day et al., 1980b) is also related to O^6-methylguanine repair capability, and this ability or inability is designated **M**ethyl **Ex**cision positive (Mex^+) or **M**ethyl **Ex**cision negative (Mex^-), respectively (Salker and Strauss, 1981). These data suggest that CENU resistance, cross-link formation, reactivation of MNNG-damaged adenovirus, and O^6-methylguanine repair are closely associated (Erickson, 1991).

The purified *E. coli* O^6-alkylguanine-DNA alkyltransferase prevents the formation of BCNU cross-links in damaged M13mp9 DNA <u>in vitro</u> (Robbins et al., 1983). This bacterial counterpart of MGMT quenchs cross-

link formation when incubated with the damaged template immediately after drug removal. However, increasing the interval between BCNU treatment of DNA and protein incubation by several hours allows cross-link formation (Robbins et al., 1983). These data suggest that the alkyltransferase protein repairs cross-link precursors, however, once the cross-links are formed, the protein is no longer effective. Experiments using a variety of CENU damaged templates and the human alkyltransferase MGMT further substantiate these observations (Brent, 1984, 1985a). Cells with high levels of MGMT activity are more resistant to CENU cytotoxicity than cells with little or no MGMT activity (Brent et al., 1985b, Yarosh et al., 1985; Robbins et al., 1983).

The cross-link precursors O^6-chloroethylguanine and O^6, N^1-ethanoguanine appear to be substrates for MGMT (Brent et al., 1985a; 1988; 1990). Interaction with the cyclized intermediate results in a covalent cross-linking of the MGMT protein to DNA via the N-1 position of guanine (Gonzaga et al., 1990). Repair of the O^6-chloroethyl monoadduct, or the formation of the DNA-MGMT cross-link quenchs DNA interstrand cross-link formation (Brent et al., 1983a; 1988; 1990; Gonzaga et al., 1990). There appears to be no regeneration of the alkylated MGMT molecule and recovery of functional protein could not be distinguished from *de novo* synthesis (Yarosh et al.,

1986). The repair of O^6-alkylguanine by MGMT is stoichiometric; one MGMT molecule repairs one alkyl lesion from the O-6 position of guanine (Pegg et al., 1983). Studies exploiting this suicidal property of MGMT to induce a temporary MER^- phenotype to CENU resistant cells will be elaborated below.

Experiments introducing various MGMT complementary DNAs into Mer^- cells demonstrated the direct cytoprotective effect of MGMT. Cytotoxicities of BCNU, ACNU, and chlorozotocin are markedly reduced by the transfection of the MGMT or *ada* cDNAs into repair deficient cells (Ling-Ling et al., 1992; Hayakawa et al.; 1992, Samson et al., 1986; Kataoka et al., 1986; Barrows et al. 1987).

Pegg et al. (1983, 1990, 1993) have proposed the following mechanism for the transfer of alkyl groups from the O-6 position of guanine to MGMT. Based on comparative cDNA sequence analysis and site-directed mutagenesis studies, the active site in MGMT has been assigned putatively to the -Pro-Cys-His-Arg-Val- motif representing the amino acid residues 144-148 of the MGMT protein (Ling-Ling et al., 1992). Site directed mutation of the cys-145 residue to valine renders the protein inactive (Ling-Ling et al., 1992). The Cys-145 residue within peptide sequence appears to be the alkyl acceptor site within MGMT (Olsson and Lindahl, 1980; Mehta et al.,

1981; Pegg et al., 1983; Ling-Ling et al., 1992). Mechanistically, the alkyl group transfer could be facilitated by the generation of a thiolate anion from the cysteine acceptor, and the adjacent arginine and histidine residues aid in the formation and stabilization of the anion. This thiolate anion could attack the alkyl group at the O-6 position of guanine yielding the native guanine and the alkylated protein. This reaction does not require any other proteins or cofactors, and has a pH optimum of 7.8-8.5 (Pegg et al., 1983). The alkyl group transfer may be a bi-molecular displacement reaction resembling the first half of the ping-pong enzyme mechanism where a functional group is transferred from the substrate to the active site of the enzyme, resulting in a covalent linkage (Pegg, 1990). The isolation of an alkylcysteine residue from MGMT incubated with methylated DNA or BG provides strong evidence for this mechanism (Pegg et al., 1983, 1993). However, the exact molecular mechanism of this reaction remains to be deduced. Site directed mutagenesis studies indicate that the histidine and valine residues in the active site are not essential for repair of methylated DNA (Ling-Ling et al., 1992). Experiments examining substrate specificity and rate constants for these mutant MGMT proteins must be performed to clarify their role in the catalytic transfer (Pegg et al., 1993).

Pharmacologic Depletion of MGMT and Reversal of CENU Resistance

Reversal of CENU resistance by DNA methylating agents.

Methylating agent-induced O^6-methylguanine lesions in DNA appears to be important in mutagenesis, carcinogenesis, and cytotoxicity. This lesion in DNA is currently the most efficient substrate for MGMT with a second order rate constant of 0.02×10^8 mol^{-1} min^{-1} (Pegg et al., 1984; Bhattacharyya et al., 1990). The O^6-methylguanine lesions in DNA produced by methylating agents are repaired by MGMT and result in the inactivation of the protein (Brent, 1986). Methylating agents are collectively referred to as indirect MGMT depleting agents because they must first react with DNA in order to provide a substrate for MGMT.

The suicidal nature of MGMT-mediated repair of O^6-methylguanine lesion has been exploited to enhance CENU cytotoxicity in resistant cells <u>in vitro</u>. Pre-treatment of resistant cells with MNNG results in the inactivation of MGMT, and thereby can potentially increase alkylating agent cytotoxicity (Waldstein et al., 1982). Pre-treatment of the CENU resistant cell lines IMR-90 and HT-29 with non-toxic doses of MNNG prior to CENU administration produces increased CNU-induced DNA

interstrand cross-links and cell killing (Zlotogorski and Erickson, 1983). Similarly, non-toxic doses of MNU, STZ and methyl methane sulfonate can also increase CNU cross-link formation and cytotoxicity in HT-29 cells (Zlotogorski and Erickson, 1984; Erickson et al., 1988). In the same cell line, mitozolamide-induced cytotoxicity and cross-link formation are enhanced by MNNG or STZ pre-treatment (Gibson and Erickson, 1985; Gibson et al., 1986). Furthermore, a non-toxic dose of STZ (2.5 mM) inhibits MGMT activity and increases cross-link formation and cytotoxicity in HT-29 cells (Futscher et al., 1989). Serial administration of temozolamide or dacarbazine and BCNU depletes MGMT and increases BCNU cytotoxicity in HT-29 cells <u>in vitro</u> (Mitchell and Dolan, 1993). However, the administration of STZ, dacarbazine, or temozolamide prior to BCNU produces negligible MGMT inactivation and growth inhibition of colon carcinoma, medulloblastoma and glioblastoma xenografts (Mitchell and Dolan, 1993; Friedman et al., 1992).

The <u>in vitro</u> effectiveness of the methylating agents in sensitizing CENU resistant cells prompted the phase I/phase II clinical testing of the combination of STZ and BCNU (Micetich et al., 1992; Panella, 1992; Willson, 1992). The dose limiting toxicities of the combination include thrombocytopenia, transient hypophosphatemia, and proteinuria. Additionally, the maximally tolerated dose

of BCNU when combined with STZ is reduced by 50% (Micetich, et al., 1992). The intrinsic cytotoxic potential of STZ and the lack of tumor selective inactivation of MGMT may be responsible for the apparent exacerbation of BCNU toxicity (Willson, 1992; Micetich, et al., 1992). Therefore, the clinical use of STZ in the inactivation of MGMT and the reversal of BCNU resistance might be limited. Furthermore, the clinical use of methylating agents as MGMT depleting agents could increase the chance of secondary malignancies because MGMT plays a pivotal role in the prevention of MNU and MNNG induced mutagenesis and carcinogenesis (Dumenco et al., 1993; Nakatsuru, et al., 1993).

Inactivation of MGMT and reversal of CENU resistance by guanine analogs.

The guanine analogs which deplete MGMT include O^6-methylguanine (MG), O^6-benzylguanine (BG), and a series of alkyl, halogenated and nucleoside derivatives of BG (Karran, 1985; Dolan et al., 1990b; Moschel et al., 1992). The free base MG was initially thought to deplete MGMT by serving as a misincorperated substrate within t-RNA (Karran, 1985). However, the incorporation of the free base into nucleic acids is not necessary to deplete MGMT activity (Dolan et al., 1985). MG can produce substantial, albeit, incomplete depletion of MGMT

activity in HeLa cells by serving as a substrate for MGMT (Dolan et al., 1985). Incubation of O^6-methyl-[^{3}H]-guanine with HeLa cell extract containing MGMT results in stoichiometric repair suggesting that MG directly interacted with MGMT (Dolan et al., 1985). The rate of repair of MG is 10^7 times slower than when contained within double stranded DNA suggesting MG is a poor substrate for MGMT (Yarosh, 1986). Pre-treatment of several resistant cell lines with millimolar concentrations of MG for periods of 4 hr or longer results in 80% depletion of MGMT and 1-2 log potentiation of CNU, CCNU, meCCNU, HeCNU, and BCNU cytotoxicities in vitro (Dolan et al., 1986; Yarosh et al., 1986, Thielmann et al., 1987, Dempke et al., 1987). Additionally, MG can also deplete MGMT activity, increase BCNU and clomesone induced cytotoxicity and enhance DNA interstrand cross-linking in HT-29 cells (Dolan et al., 1988). In contrast to its in vitro effectiveness, MG only partially inactivates MGMT and fails to inhibit the growth of HT-29 xenografts implanted in nude mice when combined with BCNU (Dolan et al, 1989). This incomplete MGMT inactivation in tumor xenografts and rodent tissues requires extremely large doses of MG (Pegg, 1990). The low affinity of MG for MGMT, lack of in vivo enhancement of CENU cytotoxicity, and the limited solubility of MG are considerable difficulties for the clinical use of MG.

Based on the premise that a benzyl group could more readily participate in a bimolecular displacement reaction than a methyl group, Dolan et al. (1990 a,b) demonstrated that the free base O^6-benzylguanine (BG) was a potent, non-toxic, irreversible inactivator of MGMT. As little as 2 μM BG can completely ablate MGMT activity within 5 min in HT-29 extracts (Dolan et al., 1990 b). Incubation of [^{3}H]-O^6-benzylguanine with extracts containing MGMT results in the liberation of stoichiometric amounts of [^{3}H]-guanine. Furthermore, the inactivation of MGMT leads to the formation of S-benzylcysteine within the protein (Pegg et al., 1993). These data suggest that BG directly interacts with MGMT, serves as a substrate for alkyl transfer, and yields a native guanine and the benzylated protein. The second order rate constant for this reaction is 600 M^{-1} s^{-1} (Pegg et al., 1993). Evidence further suggests that benzylation of MGMT induces a conformational change in the protein which may stimulate rapid proteolytic digestion of the inactive protein (Pegg, et al., 1991). Site-directed mutagenesis studies suggest that the cys-145 residue is the alkyl acceptor site and pro-140 is an integral component of the BG hydrophobic binding site within MGMT. This proline 140 residue is replaced by an alanine residue in the bacterial alkyltransferase *ada* and may be partially responsible for the differential

sensitivity of the two proteins to BG (Crone and Pegg, 1993). In addition, the sequences in the divergent 28 amino acid tail within MGMT appear to be involved in the BG binding site since the deletion of this tail reduces BG mediated inhibition of MGMT by 5 fold (Morgan et al., 1993).

The inactivation of MGMT and reversal of CENU resistance by BG has been demonstrated in numerous tumor cell lines and xenografts. Treatment of HT-29 cells with micromolar doses of BG prior to clomesone, BCNU, CCNU, or chlorozotocin administration results in the potentiation of CENU cytotoxicity (Dolan et al., 1991). Pretreatment with BG also results in the induction of BCNU interstrand cross-links, reduction in colony formation, and significant inhibition of HT-29 and SF767 xenograft growth (Dolan et al, 1990a; Mitchell et al., 1992). The enhancement of ACNU cytotoxicity or the dose modification factor (IC_{50} ACNU alone/IC_{50} BG+ACNU) is related to the functional MGMT content in a cell line or xenograft (Chen et al., 1993 a,b). The rank order of enhancement of BCNU cytotoxicity in several cell lines by BG appears to be a function of MGMT activity (Dolan et al., 1991; Chen et al., 1993 a,b). This phenomenon has been corroborated <u>in vivo</u> by Dolan et al. (1993 b) by testing the anti-tumor activity of the combination of BG+BCNU against four colon carcinoma xenografts with high, intermediate, and low

levels of MGMT activity. The effectiveness of this combination in vivo is further evidenced by the induction of partial and complete regression of medulloblastoma and glioblastoma tumors implanted subcutaneously, and intracranially (Friedman et al., 1992; Felker et al, 1993). The modulation of BCNU resistance in vivo in a colon cancer xenograft (Vaco 6) has subsequently been accomplished by repeated administrations of BG (Gerson et al., 1993). In this study, tumor bearing animals were administered BG pre- and 2 hr post-BCNU treatment. The post-BCNU administration of BG may have inactivated nascent MGMT molecules which could have maximized the formation of DNA interstrand cross-links (Gerson et al., 1993). The host toxicity of the combined use of a CENU and BG necessitates at least a 50% reduction in the dose of the CENU in many of these animal experiments. Data from histopathological examination of several rat tissues from BCNU and the combination of BG and BCNU treated rats, suggest that the dose-limiting determinants are due to gastrointestinal, splenic, and hematopoietic dysfunction (Dolan et al., 1993a).

CENU resistance modulation has predominately operated on the premise that MGMT should be initially depleted, prior to CENU exposure, in order to sensitize resistant cells to CENU cytotoxicity. However, CENU-induced cross-links continue to form for an extended period of 6-12 hr

after CENU removal (Erickson et al., 1980a). It is possible that any newly synthesized MGMT following a resistance modulator could conceivably react with a cross-link precursor and decrease the formation the lethal DNA interstrand cross-link. Therefore, in order to maximize cross-link formation and cytotoxicity, MGMT should be inactivated throughout the time course of cross-link formation. Experiments described within this dissertation address this hypothesis by determining whether extended depletion of MGMT after BG, STZ, and the combination of BG and STZ is indeed required to maximize BCNU cytotoxicity.

Role of Glutathione in CENU resistance

The majority of data strongly indicate that DNA interstrand cross-links are the predominant cytotoxic lesions and that MGMT plays a pivotal role in CENU resistance by preventing cross-link formation. However, several other mechanisms of CENU resistance have also been implicated. In four medulloblastoma cell lines, a relationship between cellular content of MGMT activity and BCNU cytotoxicity was not demonstrated (Silber, et al., 1992). Additionally, cells expressing MGMT could not be sensitized to BCNU cytotoxic effects by BG pretreatment (Silber, et al., 1992). In a subsequent study, a panel of eight human brain tumors with low to

high levels of MGMT activity were not appreciably sensitized to BCNU cytotoxicity by BG. The authors suggested that MGMT was not a primary determinant in predicting BCNU sensitivity in their cell lines and that in vivo BCNU resistance in brain tumors may be independent of MGMT content (Bobola et al., 1993). These data suggest that other mechanisms of CENU resistance may exist in MGMT expressing cell lines. A secondary mechanism of CENU resistance may involve the intracellular detoxification of CENU by glutathione (GSH).

Glutathione is present in mammalian cells at intracellular concentrations of 5-10 mM (Kosower and Kosower, 1978). This tripeptide (Glu-Cys-Gly) contains a strong nucleophilic thiol group within the cysteine residue that can act as a nucleophilic sink for numerous electrophiles such as free radicals, reactive oxygen species, and many other endogenous and exogenous compounds (Chesseud, 1979; Meister and Anderson, 1983). The xenobiotics which react with GSH include cisplatin and the DNA alkylating agents melphalan, BCNU, and nitrogen mustard (Moscow et al., 1989; Hantel., et al 1991; Evans et al., 1987). Conjugation of these electrophiles with GSH convert them to non-toxic metabolites which are transported extracellularly and degraded by proteases (Bakke, 1990). The conjugation

occurs both in enzymatic and non-enzymatic processes. The enzymatic process is mediated by a class of glutathione-S-transferases (GST) which may determine substrate specificity.

Treatment of cells with BCNU pre-incubated with GSH prior to cell exposure in vitro results in increased clonogenicity, decreased DNA interstrand cross-linking, and decreased the inhibition of DNA replication in a human glioblastoma cell line (Ali-Osman et al., 1989 a,b). Ali-Osman et al. (1989a) suggested that the conjugation of GSH with the cross-link precursor O^6-chloroethylguanine in DNA may result in the formation of 1-(O^6-deoxyguanosyl), 2-S-glutathionylethane. The formation of this conjugate may prevent the intramolecular rearrangement and subsequent reaction with the complementary base cytosine, and thereby quench cross-link formation (Ali-Osman, 1989a). The Pi and Mu isozymes of GST mediate the conjugation of BCNU with GSH followed by subsequent denitrosation of BCNU. The Pi class of isozymes appears to be responsible for detoxification of BCNU in human brain tumor cells (Ali-Osman et al., 1990), whereas the Mu isozymes mediate the conjugation of GSH and BCNU in rat brain tumor cells (Smith et al., 1989). The significance of GSH in BCNU resistance is unclear since many of the studies which addressed the role of GSH and BCNU resistance did not

assess the relative contribution of such resistance conferred by MGMT.

Sensitization of the HT-29 cell line, a MER^+ colon carcinoma cell line, to BCNU cytotoxicity by depleting GSH has been attempted by Hantel et al. (1990). This cell line contains 14-15 fold greater GSH and GST activity than its MER^- counterpart BE. Reduction in HT-29 GSH content by more than 80% with buthionine sulfoximine (BSO) (a competitive inhibitor of the rate limiting enzyme in GSH biosynthesis, gamma-gultamyl synthase) does not modify BCNU cytotoxicity. HT-29 cells contain high levels of MGMT activity, and without MGMT depletion, the significance of GSH depletion in BCNU resistance in MER^+ cells would be difficult to assess. Because MGMT is clearly an important mechanism of resistance in MER^+ cells, any secondary consequence of GSH depletion would have been masked by MGMT repair function. In order to partially resolve this issue, an experiment simultaneously targeting MGMT and GSH depletion is described in Appendix I.

CHAPTER III

PROLONGED DEPLETION OF O^6-METHYLGUANINE DNA METHYLTRANSFERASE ACTIVITY (MGMT) FOLLOWING EXPOSURE TO O^6-BENZYLGUANINE WITH OR WITHOUT STREPTOZOTOCIN ENHANCES 1,3 BIS(2-CHLOROETHYL)-1 NITROSOUREA SENSITIVITY IN VITRO

Pretreatment of resistant cells with MGMT depleting agents prior to CENU exposure successfully inactivates MGMT, enhances DNA cross-link formation, and increases cytotoxicity (Zlotogorski and Erickson, 1984; Dolan et al., 1986; Dolan et al., 1990 a,b). However, recent observations suggested that maximal sensitization of CENU resistant cells might require a more extended depletion of MGMT following BCNU (Dolan et al., 1990; Gerson et al., 1993). The observation that the DNA interstrand cross-link precursor O^6, N^1 ethanoguanine is stable for greater than 8 hr following CENU removal, suggests the potential for cross-linking exists for extended periods (Brent, 1985b). Indeed, cross-links produced by several CENU continue to form for 6-12 hr post-drug exposure and persist for at least 24 hr in the MGMT deficient cell lines (Ewig and Kohn, 1977; Erickson et al., 1980a). Furthermore, when BG is employed as the BCNU resistance

modulator, optimal enhancement of BCNU cytotoxicity requires pre- and post-BCNU exposure of cells to BG (Dolan et al., 1990; Gerson et al., 1993). The administration of BG following BCNU treatment may inactivate nascent MGMT molecules. Additionally, this inactivation could prevent the repair of the stable cross-link precursors (Gerson et al., 1993). Therefore, inactivation of MGMT for prolonged periods throughout the time-course of cross-link formation would be expected to maximize cross-link formation and cytotoxicity in MGMT expressing cells. However, no attempt has been made to compare the enhancement of CENU cytotoxicity produced by a single dose of BG with repeated administration of BG, and thereby substantiate the aforementioned conclusions. Therefore, the experiments described in this chapter attempt to determine whether extended depletion of MGMT activity is required for maximal enhancement of BCNU cytotoxicity by BG or STZ in resistant cells.

In this chapter two pretreatment protocols were used to examine whether extended depletion of MGMT was required to maximize BCNU cytotoxicity. The enhancement of BCNU cytotoxicity and the recovery of MGMT activity were measured following BG (10-100 μM) and the combination of BG (10 μM) and STZ (1.0 mM) <u>in vitro</u>. In the three drug combination of BG+STZ+BCNU, BG can deplete MGMT allowing a greater number of STZ induced O^6-

methylguanine lesions to form which can deplete MGMT for an extended period. The combination of BG+STZ produced a more extended depletion of MGMT for 24 hr and a greater enhancement of BCNU cytotoxicity than when BG or STZ were used alone. If the mechanism by which STZ increased BG+BCNU cytotoxicity was by producing a more prolonged depletion of MGMT, then higher doses of BG (10-100 μM) which can deplete MGMT for 24 hr should also produce a comparable enhancement of BCNU cytotoxicity. A 100 μM dose of BG produced complete inactivation of MGMT for greater than 24 hr and the greatest enhancement of BCNU cytotoxicity. The studies suggest that the greatest BCNU sensitization is reached when MGMT activity was depleted prior to, and for at least 24 hours following BCNU administration. Moreover, these data suggest that the design of clinical strategies targeting MGMT inactivation should consider prolonged inhibition of MGMT activity to increase the likelihood of anti-tumor responses.

Materials and Methods

Cell Culture

HT-29 cells were cultured in Eagle's minimum essential medium, supplemented with 10% bovine calf serum, 62.5 mM HEPES buffer, 1 mM glutamine, 1 mM sodium pyruvate, 1 X vitamin B12, 1 X non-essential amino acids (Hazelton) and 0.05 mg/ml gentamicin. Cells were maintained in log phase at 37°C in 95% air, 5% CO_2 atmosphere.

Colony Formation Assay

HT-29 cells were seeded at 150, 300, 1000, 3000, and 10,000 cells per T-25 mm^3 flask and incubated for 11-14 hr to allow for complete attachment. When testing the cytotoxicity of the various combinations of BG, STZ, and BCNU, triplicate flasks were individually treated with BG, STZ and/or BCNU. BG, STZ, and BCNU were dissolved in dimethylsulfoxide, 0.9% NaCl, and 95% ethanol, respectively. The administration of dimethylsulfoxide (1.0%) for 3 hr, or ethanol (1.0%) for 1 hr did not produce greater than 2-3 % inhibition of colony formation. Cells were cumulatively exposed to BG for 3 hr, STZ for 2 hr, and BCNU for 1 hr. In assessing the cytotoxicity induced by BG+BCNU, cells were treated with BG (10-100μM) for 2 hr prior to a 1 hr exposure to BCNU. Following treatment, drug containing medium was aspirated

and replaced with fresh medium. After 9-10 days, cells were fixed in 100 % methanol, stained with a buffer containing 2.31 mg/ml methylene blue, 2.5 mM Na_2HPO_4, 1.33 mM KH_2PO_4, and colonies were counted. The survival values plotted for the combinations of BG+BCNU, STZ+BCNU, and BG+STZ+BCNU were normalized to BG, STZ and BG+STZ alone induced cell killing. Survival curves show the mean and the standard deviation of three or more independent experiments. A minimum of triplicate determination of cytotoxicity were made within each experiment.

Measurement of MGMT activity

The assay for MGMT activity used in this study was developed by Wu et. al. (1987) and subsequently modified in our laboratory (Futscher et al., 1989; Pieper et al., 1991). Cultured cells were resuspended at a concentration of approximately $3x10^7$ cells/ml in assay buffer (50 mM Tris pH 8, 1 mM DTT, 1 mM EDTA, 5% glycerol), quickly frozen in dry ice, and stored at -80°C for subsequent measurement of MGMT activity. The effects of freezing and thawing of cells on MGMT activity will be discussed below.

Due to exonuclease activity of the Klenow fragment of DNA polymerase, the radiolabelling of the O^6-methylguanine containing oligomer used in this study was catalyzed by the Stoffel fragment of the Taq DNA polymerase. The 18

base pair oligomer containing an O^6-methylguanine lesion was radiolabelled by filling in the 3' recessed end with thymidine 5'-[α-^{32}P] triphosphate (TTP) (Amersham, Arlington Heights, IL). 20 pmol of probe were incubated with 10 units of Stoffel Taq DNA polymerase (Perkin Elmer/Cetus, Norwalk, CT), 50 μCi of TTP (specific activity 3000 Ci/mmol), 1.5 mM $MgCl_2$, 1X Taq buffer at 42°C for 1 hr. The labeling reaction was terminated with sequential extraction with phenol:chloroform:isoamyl alcohol (PCI) and chloroform:isoamyl alcohol (CI). Subsequently the DNA was precipitated in 70% ethanol, 73.0 mM sodium acetate and 20 μg of t-RNA.

In measuring MGMT activity, the radiolabelled oligomer was incubated with 10 μg of total cellular protein (2 hr, 37°), PCI extracted, digested with Pvu II (1 hr, 37°) (Gibco BRL, Gaithersburg, MD), and electrophoresed on a 20% denaturing polyacrylamide gel. The extent of restriction enzyme cleavage of the radiolabelled 18 mer to an 8 mer by Pvu II is directly proportional to MGMT activity (Wu et al., 1987). The radioactivity was quantitated on a Betagen Betascope 603 blot analyzer. The % probe cleaved = (CPMs 8 mer/ CPMs 18 mer + CPMs 8 mer) X 100. Measurement of MGMT activity following treatment with BG and STZ was normalized to untreated control values. Each data point represented the mean and the standard deviation of three independent experiments.

Freezing and thawing of cells did not result in appreciable loss of MGMT activity. Incubation of the radiolabelled 18 mer with 25 μg of extracts freshly prepared HT-29 extract resulted in 76.4 +/- 2.16 % probe cleaved following Pvu II digestion. Incubation of 25 μg frozen cell extract for 24 hr at -80°C with the O^6-methylguanine containing DNA resulted in 76.0 +/- 4.2 % probe cleaved following Pvu II digestion.

S1 nuclease analysis of steady state MGMT mRNA levels
Labeling of the MGMT S1 probe

A PCR-generated MGMT cDNA was subcloned into the pGEM -3Z f- vector (Promega, Madison, Wi) and designated pMGMT (Pieper et al., 1990). pMGMT (10 μg) was digested with 10 units of Hinl I (Promega), and electrophoresed on a 1% agarose gel. The 2.4 kb Hinl I fragment was excised from the gel and purified by centrifugation through silanized glass wool at 12,000 X g. The filtrate was extracted with phenol, and PCI, and then precipitated with ethanol. The purified DNA was incubated with 5 units of Klenow DNA polymerase (Promega) and 10 μCi of deoxycytidine 5'-[α-^{32}P] triphosphate (Amersham) (6000 Ci/mmol) at room temperature for 15 min. The labelling reaction was terminated by extracting once with PCI, once with CI extraction, followed by precipitation with ethanol. This probe protects 570 nucleotides of MGMT mRNA.

S1 nuclease protection assay

Excess labelled MGMT S1 probe was incubated with 40μg of cytoplasmic RNA in 0.4 M NaCl, 40 mM 1,4-piperazinediethanesulfonic acid (pH 6.8), 2 mM EDTA and 80% formamide. The hybridization mixture was denatured at 68^{0}C for 15 min, immediately transferred to a 53^{0}C bath, and incubated >16 hr. Subsequently, the nucleic acid was digested with 400 U of S1 nuclease (Boehoringer Mannheim, Philadelphia, PA) at 37°C for 1 hr, extracted with PCI, precipitated with ethanol, and electrophoresed on a 4% denaturing polyacrylamide gel. The gel was fixed in 10% acetic acid for 15 min, soaked in water for 15 min, dried at 80^{0}C for 1 hr, and autoradiographed.

The probe was determined to be at least 100 fold in excess of MGMT mRNA represented within 40 μg of HT-29 RNA by comparing the intensity on the autoradiograph of 1:100 dilution of the radiolabelled probe alone to the intensity of 100 fold greater amount of probe incubated with 40 μg of RNA. As determined by densitometric analysis, the intensity of the probe alone control was 11.70 % greater in intensity than that was measured in 40 μg of RNA isolated from untreated HT-29 cell (Figure 3.6). Therefore, the probe used in these experiments was at least 100 fold in excess.

Results

Dose dependent depletion of MGMT activity by BG.

To measure MGMT activity, a ^{32}P-end-labeled 18 base pair oligomer containing an O^6-methylguanine lesion in the Pvu II restriction site was incubated with 10 μg of whole cell sonicate protein from control and BG treated HT-29 cells. Repair of O^6-methylguanine by MGMT allowed Pvu II cleavage of the oligomer to an 8 bp ^{32}P-labeled fragment. The percentage of restriction enzyme cleavage was directly proportional to MGMT activity (Wu et al., 1987). The 10 μg of HT-29 cell extract was used in the measurement of MGMT activity to maximize the sensitivity of the assay. This amount of protein was at the saturation threshold of the assay (Appendix II). The experiments described in this dissertation were designed to measure decreases and not increases in MGMT activity following drug treatment. Therefore, small decreases in MGMT activity could reliably be measured at the maximal sensitivity of the assay in HT-29 cells using this protein concentration. Exposure of HT-29 cells to BG (0.05-25 μM) for 3 hr depleted MGMT activity in a dose dependent fashion with an approximate dose for 50% inhibition of 150 nM (Figure 3.1). To insure maximal inhibition of MGMT activity, a 10 μM dose of BG was

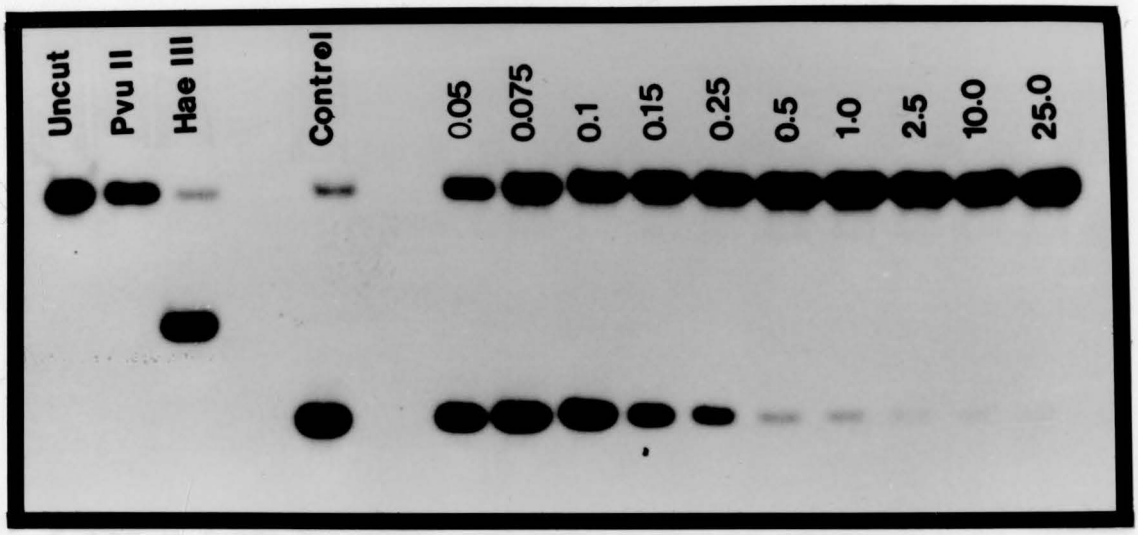


Figure 3.1:

The effect of O^6-benzylguanine (BG) on MGMT activity in HT-29 cells.

Cells were exposed for 3 hrs with 0.05-25 μM BG. O^6-methylguanine containing probe was incubated with 0 μg protein (lane 1), 0 μg protein and digested with Pvu II (lane 2), 0 μg protein and digested with Hae III (lane 3), 10 μg of total cellular protein from untreated (lane 4) or BG treated HT-29 cells (lanes 5-14) and digested with Pvu II. Extent of Pvu II digestion is proportional to MGMT activity.

chosen for BCNU sensitization studies.

Comparison of the cytotoxicity induced by the combination of BG+BCNU, STZ+BCNU, BG+STZ+BCNU.

Figure 3.2 illustrates the enhancement of BCNU cytotoxicity by BG alone, STZ alone, or the combination of BG+STZ. BG alone, STZ alone, or combination of BG (10μM) and the three doses of STZ were non-cytotoxic to HT-29 cells (Table 3.1). Therefore, the observed enhancement of BCNU cytotoxicity by the various pretreatment regimen was indeed synergistic. Treatment of HT-29 cells with 10 μM BG for 2 hr prior to a 1 hr exposure to BCNU resulted in an increase in the toxicity of 100μM BCNU by approximately 0.5 log (Figure 3.2). Administration of STZ at the non-toxic doses of 0.25-1.0 mM for 2 hr prior to BCNU exposure did not appreciably modify BCNU cytotoxicity. However, the addition of STZ at the non-toxic doses of 0.25-1.0 mM to BG+BCNU increased cytotoxicity in a dose dependent manner. HT-29 cells were exposed to BG (10 μM) for 1 hr, followed by STZ [0.25 mM (A), 0.5 mM (B), 1.0 mM (C)] for 1 hr, and subsequently to a 1 hr exposure to BCNU. These cells received a 3 hr, 2 hr, 1 hr, exposure to BG, STZ, and BCNU, respectively. The addition of non-cytotoxic doses of STZ (0.25, 0.5, 1.0 mM) to BG+BCNU resulted in approximately 0.5-1.0 additional log enhancement of BG+BCNU cytotoxicity. The enhancement of BCNU

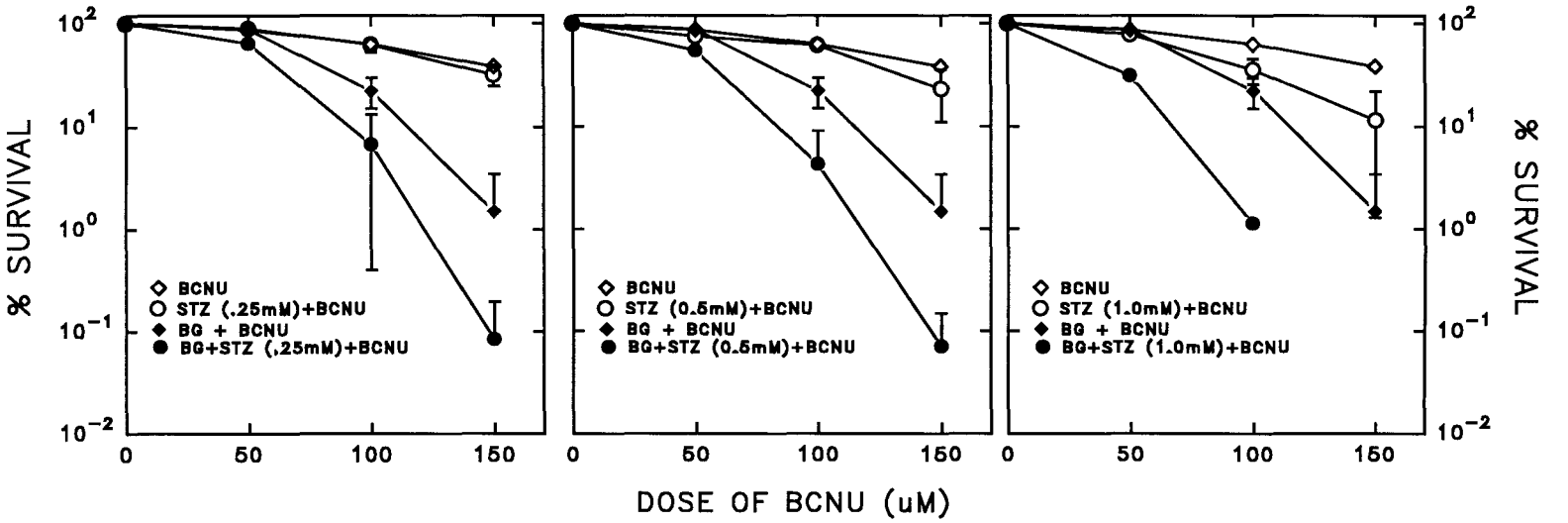


Figure 3.2:

Comparison of the cytotoxicity induced by BG+BCNU, STZ+BCNU, or BG+STZ+BCNU.

HT-29 cells were treated with BCNU alone, or STZ (0.25, 0.50, 1.0 mM) + BCNU, or BG (10 μM)+BCNU, or BG (10 μM) + STZ (0.25, 0.50, 1.0 mM) + BCNU. Cells were exposed to BG for 2 hrs and STZ for 1 hr prior to a 1 hr exposure to BCNU. Identical survival curves for BCNU alone and BG+BCNU are reproduced in panels A, B and C. Panel A, B, and C, illustrate the enhancement of BG+BCNU cytotoxicity by STZ at 0.25, 0.5, and 1.0 mM respectively. Each data point represents the mean and the standard deviation of three or more independent experiments. However, the data point depicting the cytotoxicity produced by BG(10 μM) + STZ (1 mM) + BCNU (100 μM) represents the mean of two independent experiments.

Table 3.1:

Cell survival[a] following the various pretreatment regimens used to enhance BCNU cytotoxicity

	% Survival
BG (10.0 μM)[b]	92.10 +/- 6.50
STZ (0.25 mM)[b]	108.2 +/- 11.6
STZ (0.50 mM)[b]	112.2 +/- 17.2
STZ (1.00 mM)[b]	107.9 +/- 12.7
BG (10.0 μM) + STZ (0.25 mM)[c]	107.3 +/- 6.80
BG (10.0 μM) + STZ (0.50 mM)[c]	104.1 +/- 1.10
BG (10.0 μM) + STZ (1.00 mM)[c]	102.1 +/- 11.9

[a] The survival values reported in this table indicate the percentage of cells surviving +/- the standard deviation after treatment with the various regimens. [b] The cytotoxicity scores for STZ, BG alone are normalized to untreated control values. [c] The combination cytotoxicity values are normalized to scores obtained with BG alone, and therefore reflect the cytotoxicity produced by STZ when combined with BG.

cytotoxicities, as reflected in the doses of BCNU required to kill 90 % of seeded cells (IC90), is illustrated in Table 3.2. The addition of STZ at a dose of 1.0 mM to the BG+BCNU regimen resulted in 1.86 fold reduction in IC90 dose of BCNU.

Resynthesis of MGMT activity following treatment with BG, STZ, and BG+STZ.

When STZ was used as the sole modulator of CENU resistance, the formation of BCNU induced interstrand cross-links and the enhancement of cytotoxicity were related to the regeneration rate of MGMT activity (Erickson et al., 1988; Pieper et al 1991). As the interval between STZ and CENU was increased, MGMT activity recovered, and the potentiation of cross-link formation and cytotoxicity decreased. These data suggest that resynthesis of MGMT following STZ might be an important determinant in CENU resistance. Based on these observations, I hypothesized that the mechanism by which STZ enhanced the toxicity of BG+BCNU was by producing a more extended depletion of MGMT. Figure 3.3 illustrates the regeneration of MGMT activity following treatment of cells with BG (10 μM), or STZ (1.0 mM), or BG+STZ. Adhering to the treatment strategy used in the survival assays, HT-29 cells were exposed to BG for 3 hr and STZ for 2 hr.

Table 3.2:

The IC90 doses[a] of BCNU after treatment with BG, STZ, and the combination of BG+STZ

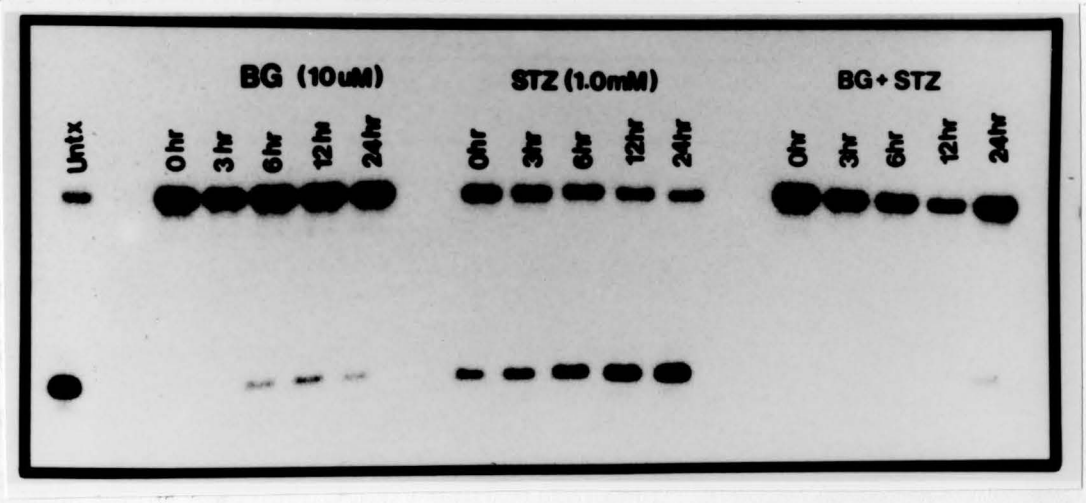
BCNU alone	> 150 μM
BCNU + BG (10.0 μM)	117.5 μM
BCNU + BG (10.0 μM) + STZ (0.25 mM)	92.5 μM
BCNU + BG (10.0 μM) + STZ (0.50 mM)	82.5 μM
BCNU + BG (10.0 μM) + STZ (1.00 mM)	63.1 μM

[a] The concentration of BCNU required to inhibit the growth of 90 % of seeded cells. The values depicted in this table were extrapolated from the data illustrated in figure 3.2.

Figure 3.3:

MGMT activity in HT-29 cells following treatment with BG alone (10 μM), or STZ alone (1.0 mM), or BG (10 μM) + STZ (1.0 mM).

Following exposure of cells to BG alone for 3 hrs, STZ alone for 2 hrs or the combination of BG + STZ, drug containing medium was aspirated and replaced with fresh media. Cells were harvested 0 hr, 3 hr, 6 hr, 12 hr, 24 hr following drug removal. Panel A depicts MGMT activity in HT-29 cells following drug treatment. Quantitation of the results within panel A is shown in panel B. MGMT activity is expressed as a percentage of activity measured in untreated HT-29 cells. Each data point represents the mean and the standard deviation of three independent experiments.


BG (10uM)
STZ (1.0mM)
BG+STZ
Untx
0hr
3hr
6hr
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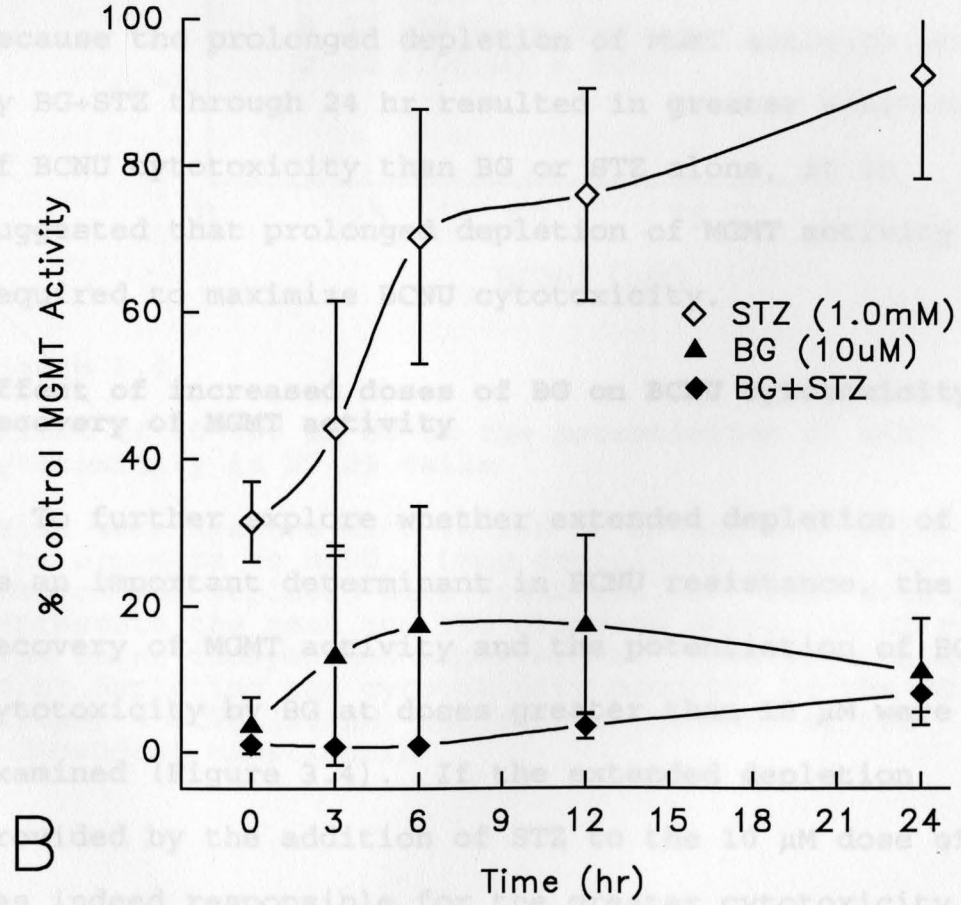
A


% Control MGMT Activity
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
Time (hr)
STZ (1.0mM)
BG (10uM)
BG+STZ

B

Subsequently, the drug containing medium was aspirated, replaced with fresh media, and the cells were allowed to recover for 0, 3, 6, 12, 24 hr following drug removal. At each time point, cells were harvested to assess MGMT activity (Figure 3.3 A,B) and mRNA levels (Figure 3.6). Cells exposed to 1 mM STZ alone gradually recovered MGMT activity reaching near control levels by 24 hr. In contrast, cells exposed to BG (10 μM) recovered approximately 15% of control activity within 6 hr, and essentially remained at that level through 24 hr. Upon sequenced administration of BG and STZ, MGMT activity was completely inhibited essentially for 24 hr. Because the prolonged depletion of MGMT activity produced by BG+STZ through 24 hr resulted in greater enhancement of BCNU cytotoxicity than BG or STZ alone, it is suggested that prolonged depletion of MGMT activity is required to maximize BCNU cytotoxicity.

Effect of increased doses of BG on BCNU cytotoxicity and recovery of MGMT activity

To further explore whether extended depletion of MGMT is an important determinant in BCNU resistance, the recovery of MGMT activity and the potentiation of BCNU cytotoxicity by BG at doses greater than 10 μM were examined (Figure 3.4). If the extended depletion provided by the addition of STZ to the 10 μM dose of BG was indeed responsible for the greater cytotoxicity, then

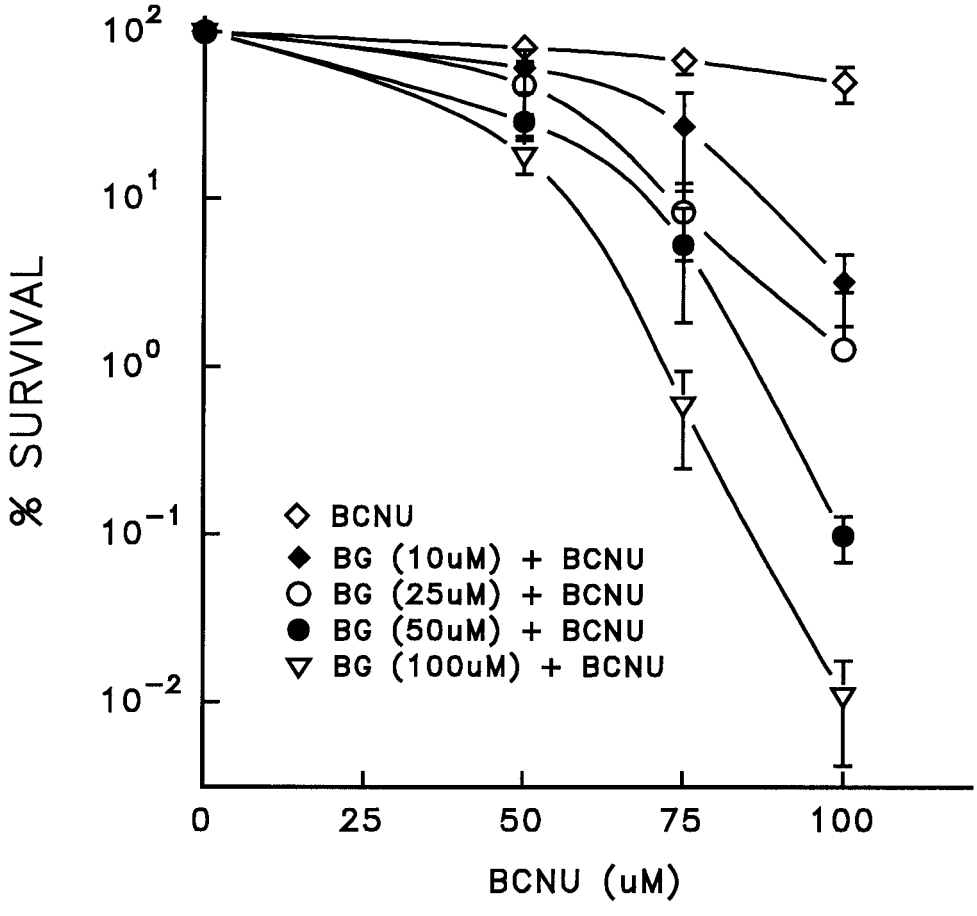


Figure 3.4:

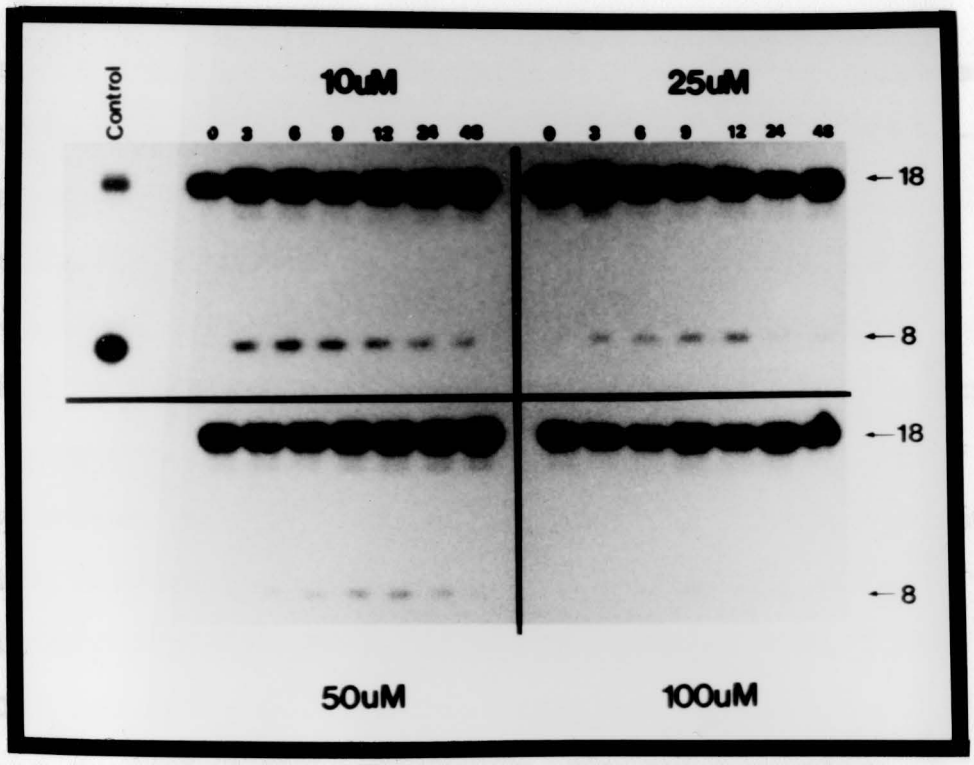
Effect of 10-100 μM BG on the potentiation of BCNU cytotoxicity in HT-29 cells

Cells were treated with 10-100 μM BG for 2 hr prior to a 1 hr exposure to BCNU. Drug containing medium was removed and replaced with fresh medium. Each data point represents the mean and the standard deviation of results from three independent experiments. However, the data point depicting the cytotoxicity produced by the BG (100μM) + BCNU (100μM) represents the mean of two independent experiments.

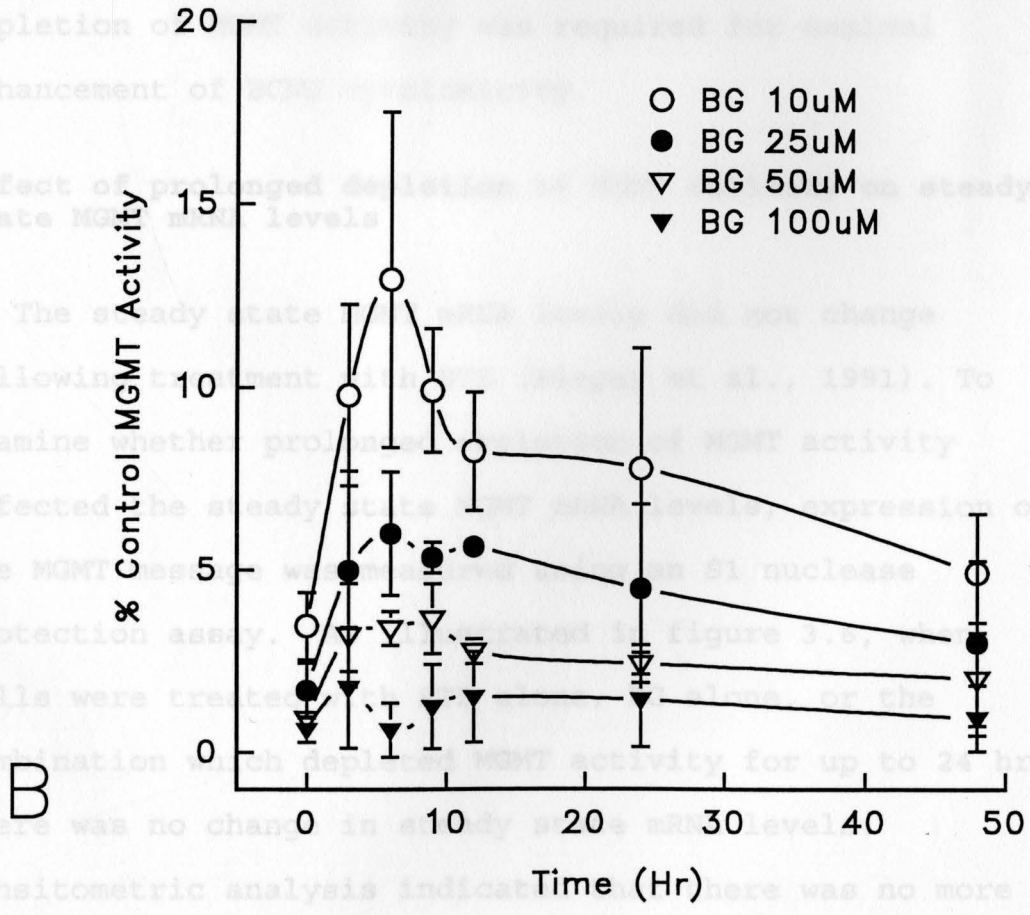
Figure 3.5:

MGMT activity in HT-29 cells following treatment with BG (10-100μM).

Cells were treated with BG (10-100 μM) for 3 hr, replaced with fresh medium, and harvested at 0, 3, 6, 9, 12, 24, and 48 hr after drug treatment. Panel A illustrates the MGMT activity in HT-29 cells following treatment with 0, 10, 25, 50, and 100 μM BG. Within each quadrant of panel A, lanes 1 through 7 represent MGMT activity at 0, 3, 6, 9, 12, 24, 48 hr following BG removal. Panel B represents quantitation of results within panels A, where MGMT activity is expressed as a percentage of MGMT activity within untreated HT-29 cells. Each data point represents the mean and the standard deviation of three independent experiments.


Control
10uM
25uM
0 3 6 9 12 24 48
0 3 6 9 12 24 48
18
8
18
8
50uM
100uM

A


BG 10uM
BG 25uM
BG 50uM
BG 100uM
% Control MGMT Activity
Time (Hr)
0
5
10
15
20
0
10
20
30
40
50

B

increased doses of BG which deplete MGMT for an extended period should produce comparable cytotoxicity. When HT-29 cells were pretreated for 2 hr with 10-100 μM BG and subsequently challenged with BCNU, the potentiation of BCNU-induced cell kill increased in a dose dependent fashion (Figure 3.4). When the recovery of MGMT activity was measured following exposure to 10-100 μM BG, MGMT activity was also inhibited in a dose and time dependent fashion (Figure 3.5). At a 100 μM dose of BG, which completely depleted MGMT for 48 hr, greater than 3 logs of BCNU induced cell kill was observed. These results further supported the hypothesis that the extended depletion of MGMT activity was required for maximal enhancement of BCNU cytotoxicity.

Effect of prolonged depletion of MGMT activity on steady state MGMT mRNA levels

The steady state MGMT mRNA levels did not change following treatment with STZ (Pieper et al., 1991). To examine whether prolonged depletion of MGMT activity affected the steady state MGMT mRNA levels, expression of the MGMT message was measured using an S1 nuclease protection assay. As illustrated in figure 3.6, when cells were treated with STZ alone, BG alone, or the combination which depleted MGMT activity for up to 24 hr, there was no change in steady state mRNA levels. Densitometric analysis indicated that there was no more

than 1.30 % difference between any of the treatment groups and untreated control. Additionally, in HT-29 cells depleted of MGMT activity for 48 hr with 100μM BG, MGMT mRNA levels were unaltered (Figure 3.7). Quantitation of these results demonstrated that there was no greater than 14.3 % difference between measurements made in control and BG treated cells.

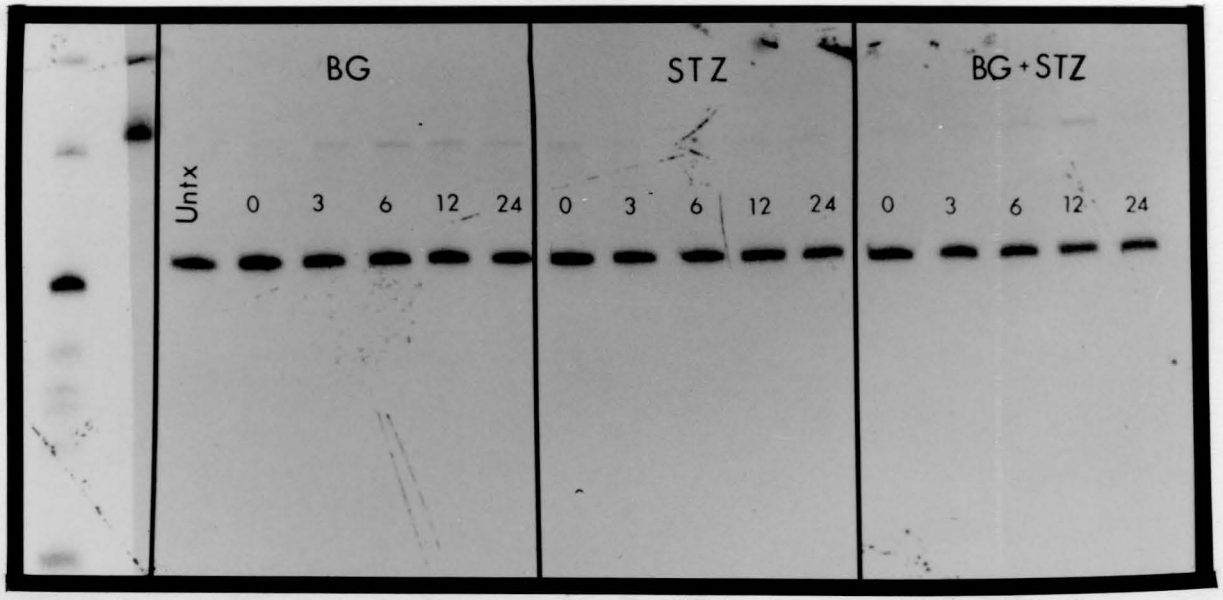


Figure 3.6:

Steady state mRNA levels following BG (10 μM), STZ (1 mM) or BG+STZ in HT-29 cells.

Cells collected in the MGMT regeneration experiments were split for the determination of MGMT activity and mRNA levels. 40 μg of cytoplasmic RNA from treated and untreated HT-29 cells were hybridized with excess probe and digested with S1 nuclease resulting in the correct 570 nucleotide protection of the MGMT mRNA. Lane designations proceeding from left to right are the following: lane 1, molecular size markers; lane 2, 1:100 dilution of undigested S1 probe; lane 3, level of MGMT mRNA in untreated cells; lanes 4-8, mRNA levels in BG (10 μM) treated cells, lanes 9-13, mRNA levels in cells treated with STZ (1 mM), lanes 14-18, mRNA levels in cells treated with the combination of BG+STZ. The numbers indicated above lanes 4-18 depict the time of cell harvest in hours following drug removal.

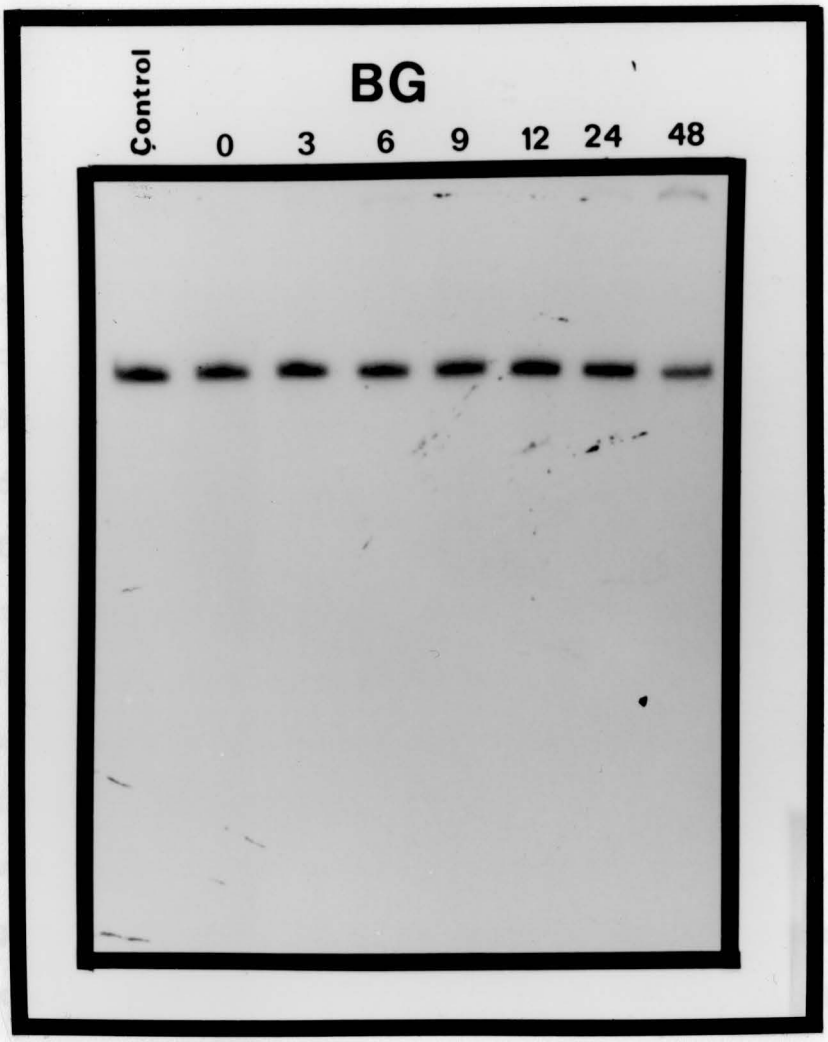


Figure 3.7:

Steady state MGMT mRNA levels following a 100 μM dose of BG

Lane 1 represents the levels of mRNA in untreated HT-29 cells. Lanes 2-8 represents the level of MGMT mRNA from cells treated with BG at a dose of 100 μM for 3 hr and harvested at the indicated times (in hours).

However, this recovery could be prevented for 24 hr by the addition of a 1.0 mM dose of STZ, or for 48 hr by increasing the dose of BG to 100 μM. The prolonged inhibition of MGMT produced by either STZ addition, or by BG (100μM) resulted in a greater enhancement of BCNU cytotoxicity than the 10μM dose of BG. These observations may be explained in the following fashion. When STZ was added to BG (10μM), the STZ induced O^6-methylguanine lesions persisted in DNA providing substrates for MGMT, and resulted in the repair-mediated inactivation of the protein for prolonged periods. By increasing the dose of BG to 100μM, the residual amounts of BG remaining within cells, after drug removal, probably provided ample concentrations of the compound to inactivate MGMT for extended periods. As a consequence of this prolonged inactivation, the repair of the CENU-induced chloroethyl monoadducts was prevented, resulting in increased levels of interstrand cross-links, and BCNU cytotoxicity. Collectively, the data suggest that extended depletion of MGMT activity is an important determinant in the reversal of CENU resistance, and that chemotherapeutic regimens targeting the inactivation of MGMT activity should be optimized such that MGMT activity is depleted for prolonged periods following CENU administration.

Pieper et al. (1991) observed that the resynthesis of

MGMT activity after treatment with STZ was not accompanied by changes in steady state MGMT mRNA levels. In order to ascertain whether a sustained inhibition of MGMT activity would induce a compensatory change in mRNA levels, a sensitive S1 nuclease protection assay was developed to measure changes in mRNA levels. There was no change in steady state mRNA levels when MGMT activity was depleted for 24 hr with the combination of BG+STZ, or for 48 hr with 100μM BG. These data demonstrated that during the period of prolonged depletion of MGMT activity, the expression of MGMT mRNA was not inducible. The observed regeneration of MGMT activity following STZ or BG treatment was probably the result of a slow nascent translation of steady state MGMT mRNA because MGMT was not reactivated after alkyl transfer (Yarosh et al., 1986), and because MGMT repletion was dependent on RNA and protein synthesis (Yarosh et al., 1986; Gerson, et al., 1988).

Additionally, STZ mediated reversal of CENU resistance necessitated a 2.5 mM dose of STZ prior to BCNU administration <u>in vitro</u> (Futscher et al., 1989). Because 2.5-10 fold lower doses of STZ (0.25 mM-1.0 mM) when combined with BG+BCNU effectively sensitized resistant cells <u>in vitro</u>, a clinical regimen using reduced doses of STZ in conjunction with BG+BCNU might attenuate the clinical toxicity observed in the STZ+BCNU

clinical trial (Micetich et al., 1992). If BG were to be used in combination with STZ, then a greater and prolonged inhibition of MGMT activity could be achieved. Moreover, the combined use of BG and STZ as cooperative modulators of CENU resistance could increase the possibility of an anti-tumor response compared to the use of either agent alone. The conclusions made in this chapter are further tested in the following chapter where the enhancement of BCNU cytotoxicity is examined under conditions of complete inhibition, partial or complete recovery of MGMT activity by 24 hr.

CHAPTER IV

EXTENDED DEPLETION OF O^6-METHYLGUANINE DNA METHYLTRANSFERASE ACTIVITY FOLLOWING O^6-BENZYL-2'-DEOXYGUANOSINE, OR O^6-BENZYLGUANINE COMBINED WITH STREPTOZOTOCIN TREATMENT ENHANCES 1,3 BIS(2-CHLOROETHYL)-1-NITROSOUREA CYTOTOXICITY

In the previous chapter, cells recovered only 10-15% of control activity by 6 hr following treatment with a 10 μM dose of BG. Increasing the dose of BG from 10 to 100 μM, or combining BG (10μM) with STZ prevented this recovery and produced a greater enhancement of BCNU cytotoxicity. From these data, it was suggested that extended depletion of MGMT was required to maximally enhance BCNU cytotoxicity in resistant cells. However, one may question whether the inhibition of only an additional 10-15% of control MGMT activity for 24 hr by the combination of BG+STZ can indeed explain the greater enhancement of BCNU cytotoxicity. Therefore, a repeated wash protocol was developed to in order to more definitively determine whether the regenerative rate of MGMT activity is related to the enhancement of BCNU cytotoxicity by BG or STZ. The regeneration of MGMT

activity in the VACO 6 cell line after BG administration was dramatically altered by washing of cells with serum containing media (Gerson et al., 1993). Washed cells rapidly recovered to control levels of MGMT activity, whereas unwashed cells remained depleted (Gerson et al., 1993). In this chapter experiments will be presented which use the repeated washing of cells to modulate the recovery rate of MGMT activity, and to evaluate the potentiation of BCNU cytotoxicity induced by regimens which provide complete inhibition, partial or complete recovery of MGMT activity by 24 hr. The regenerative rates of MGMT activity and the enhancement of BCNU cytotoxicity with and without washing were measured following BG, STZ, O^6-benzyl 2'-deoxyguanosine (dBG), and the combinations of BG+STZ and dBG+STZ.

The recovery of MGMT after treatment with nucleoside derivative of BG, dBG, was studied because it was anticipated that this compound could potentially be phosphorylated, trapped within the cells, unaffected by washing, and continue to deplete MGMT for an extended period. However, as the studies presented in this chapter demonstrate, this compound behaves similar to BG in terms of MGMT inactivation and the potentiation of BCNU cytotoxicity. The data in this chapter further confirm that extended deletion of MGMT is required for maximal enhancement of BCNU cytotoxicity.

Materials and Methods

Colony Formation Assay

HT-29 cells were seeded at densities ranging from 150-100,000 cells per T-75 mm^3 flask and incubated for 12 hr to allow for complete attachment. Cells were cumulatively exposed to BG or dBG for 3 hr, STZ for 2 hr, and BCNU for 1 hr. Following treatment, drug containing medium was aspirated, replaced with an equal volume of fresh medium, and then cells were allowed to recover for 9-10 days. Alternatively, the cells were repeatedly washed with an equal volume of serum containing medium at 37°C. A single wash cycle consisted of the following manipulations: 1) Medium from each flask was aspirated and replaced with fresh medium; 2) The flasks were gently tumbled three times. This process was repeated four times. After the final wash, cells were incubated for 9-10 days, colony formation was assessed as described in the previous chapter. When testing the cytotoxicity of the combinations of BG+BCNU, dBG+BCNU, STZ+BCNU, BCNU induced cell kill was normalized to BG, dBG, STZ controls, respectively. The cytotoxicity produced by the various pretreatment regimens is illustrated in Table 4.1. STZ, BG, dBG as single agents were not toxic to HT-29 cells. However, the combinations of BG+STZ or dBG+STZ were mildly toxic. In assessing the cytotoxicity of the

Table 4.1:

Survival[a] following various pretreatment regimens used to enhance BCNU cytotoxicity

	% Survival	
	Without washing	With wash
STZ (2.5 mM)[b]	90.06 +/- 10.8	91.52 +/- 10.6
BG (100 μM)[b]	93.30 +/- 6.80	90.30 +/- 6.00
dBG (1.0 mM)[b]	98.90 +/- 1.80	92.46 +/- 14.8
BG (100 μM) + STZ (2.5 mM)[c]	68.80 +/- 5.40	77.70 +/- 6.80
dBG (1.0 mM) + STZ (2.5 mM)[c]	41.36 +/- 19.3	87.6 +/- 21.6

[a] The survival values reported in this table indicate the percentage of cells surviving +/- the standard deviation after treatment with the various regimens. [b] The survival scores for STZ, BG, or dBG alone were normalized to untreated control values. [c] The survival values for the combination of BG+STZ, or dBG+STZ were normalized to scored obtained when cells were treated with BG or dBG alone, and therefore reflect the cytotoxicity produced by STZ when combined with BG or dBG.

combinations of BG+STZ+BCNU and dBG+STZ+BCNU, the cytotoxicity produced by these combinations was normalized to the cell killing produced by BG+STZ and dBG+STZ, respectively. Therefore, the survival values reflected the synergistic enhancement of BCNU cytotoxicity by the various pre-treatment regimens. Survival histograms show the mean and the standard deviation of at least three independent experiments.

Measurement of MGMT activity

The protocol utilized in these experiments was similar to that described in chapter 3 except for one modification. In measuring MGMT activity, the radiolabelled O^6-methylguanine containing oligomer was incubated with 10 μg of total cellular protein at 37°C for 2 hr, digested with 40μg proteinase K (in the presence of 1.0 % sodium dodecyl sulfate) for 1 hr at 45°C. The proteinase K digestion was added to increase the recovery of radioactivity. After protein extraction, the DNA was digested with Pvu II (Gibco BRL, Gaithersburg, MD), and electrophoresed on a 20% denaturing polyacrylamide gel. The extent of restriction enzyme cleavage of the radiolabelled 18 mer to an 8 mer by Pvu II was directly proportional to MGMT activity (Wu et al., 1987). The radioactivity was quantitated on a Betagen Betascope 603 blot analyzer. The % probe cleaved

= (CPMs 8 mer/ CPMs 18 mer + CPMs 8 mer) X 100.

Measurement of MGMT activity following treatment with BG, dBG and/or STZ was normalized to untreated control values.

Results

Effects of the combination of BG+STZ on the recovery of MGMT activity and potentiation of BCNU cytotoxicity.

Figure 4.1 illustrates the repletion of MGMT activity in HT-29 cells following treatment with BG, STZ or the combination of BG+STZ. Consistent with the observations made in the VACO 6 cell line, the rate of repletion of MGMT activity following BG (100 μM) treatment increased upon repeated washing. Unwashed cells exhibited complete depletion of MGMT activity for 24 hr. In contrast, washed cells gradually recovered MGMT activity and reached near control levels by 24 hr. The repletion profile of MGMT activity following STZ treatment was unaffected by washing. STZ at a dose of 2.5 mM completely depleted MGMT activity for 12 hr after drug removal, and cells gradually recovered to approximately 30% of control activity by 24 hr. When cells were treated with BG and STZ in a sequential manner, the inactivation of MGMT activity was complete for 24 hr, irrespective of washing. These data suggested that when cells were depleted of MGMT with BG, prior to STZ administration, a greater number of STZ induced O^6-methylguanine lesions persisted and continued to inactivate nascent MGMT molecules for extended periods.

The striking differences in the repletion profile of MGMT activity illustrated in Figure 4.1 predict dramatic

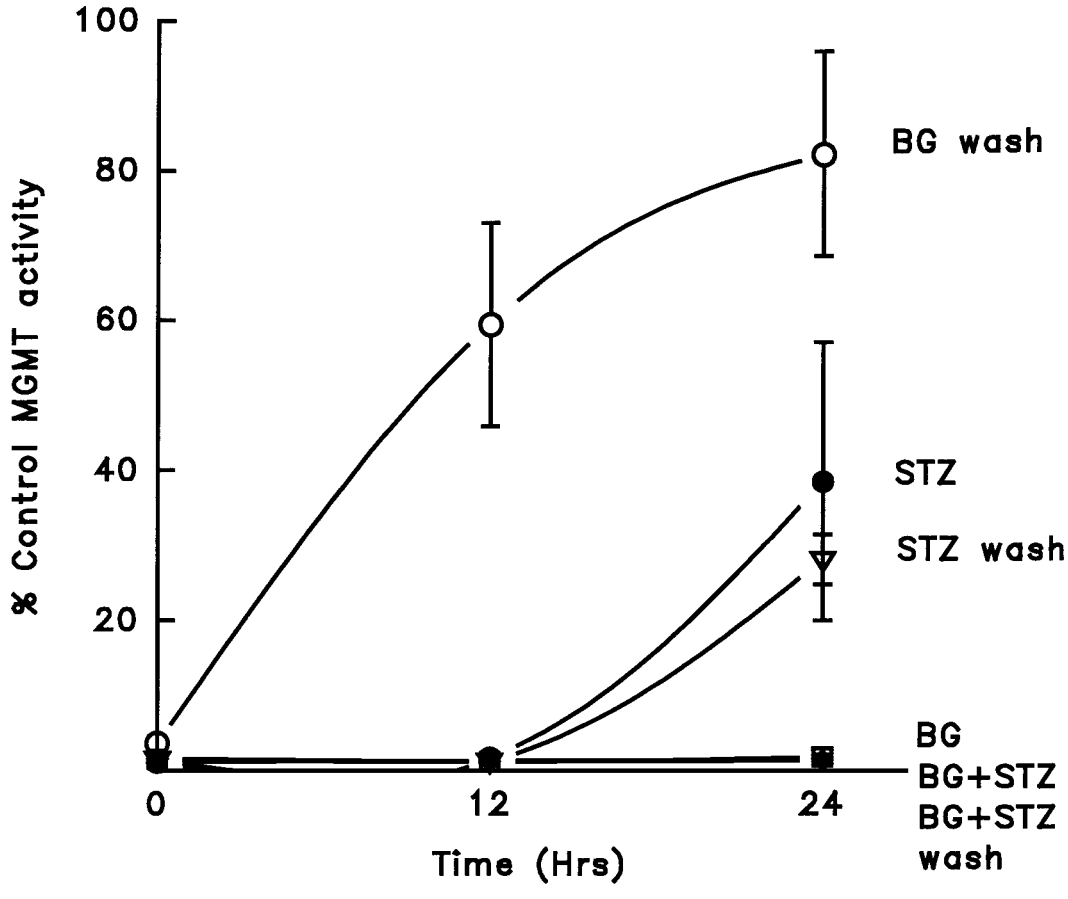


Figure 4.1:

Differential recovery of MGMT activity following BG, STZ, or the combination of BG and STZ.

HT-29 cells were exposed to BG (100 μM), STZ (2.5 mM) alone, or sequentially with the combination. Following drug treatment, some treated cells were washed as described in the "Materials and Methods" section. The cells were harvested and analyzed for MGMT activity at the indicated times after drug treatment with or without washing. Points, mean +/- SD of three independent experiments.

differences in the potentiation of BCNU cytotoxicity if extended depletion of MGMT activity is required for maximal enhancement of BCNU cytotoxicity. The rank order of the potentiation of BCNU cytotoxicity should inversely follow the repletion rate of MGMT activity. Those regimens producing the most prolonged inhibition of MGMT activity would be expected to produce the greatest cytotoxicity.

Figure 4.2 illustrates the potentiation of BCNU cytotoxicity by BG, STZ, and the combination of BG+STZ with and without washing. The combination of BG (100 μM) + BCNU (100 μM) without a wash produced approximately two to three logs of synergistic cell kill. However, cells exposed to the identical regimen and washed after BCNU exposure were dramatically less sensitive to the drug. This treatment produced less than one log of BCNU (100 μM) synergistic cell kill. The sequential administration of STZ followed by BCNU (100 μM) produced approximately 2 logs of synergistic cell kill. STZ mediated potentiation of BCNU cytotoxicity, similar to its repletion profile, was unaffected by washing. The combination of BG (100 μM) + STZ (2.5 mM) + BCNU (100 μM) produced 3-4 logs of synergistic cell kill and this cytotoxicity was not appreciably affected by washing. These data demonstrate that extended depletion of MGMT activity is required for maximal enhancement of BCNU cytotoxicity because the pre

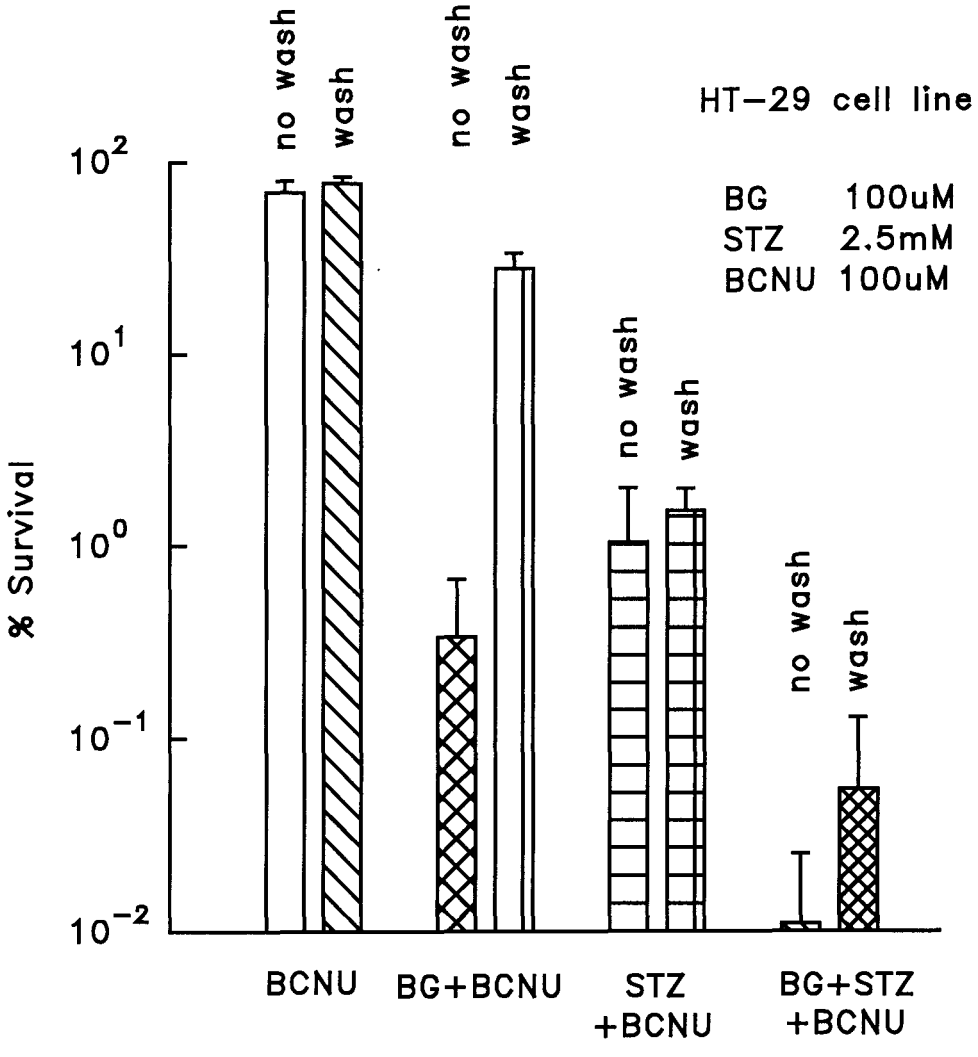


Figure 4.2:

Differential potentiation of BCNU cytotoxicity by BG, STZ, or BG+STZ, with or without washes.

Cells were sequentially exposed to BG and BCNU at a 2 hr interval, STZ and BCNU or BG,STZ and BCNU at 1 hr intervals with the indicated doses. The treatment and wash protocols are detailed in the "Materials and Methods" section. The survival histograms depict the mean +/- SD of values obtained from three independent experiments.

treatment regimens which produced the slowest recovery rate produced the greatest cytotoxicity.

Effects of the combination of dBG+STZ on the recovery of MGMT activity and the potentiation of BCNU cytotoxicity.

To further test the hypothesis that extended depletion of MGMT activity was required for optimal reversal of BCNU resistance, the repletion of MGMT activity and potentiation of BCNU cytotoxicity by the nucleoside derivative of BG, dBG was examined. This compound has 10 fold lower affinity for MGMT (Moschel et al., 1992). Figure 4.3 illustrates the repletion profile of dBG and BG at their respective IC_{100} doses (i.e. the dose producing complete inactivation in the oligonucleotide restriction assay). Unwashed HT-29 cells recovered approximately 15% of control activity by 6 hr and essentially remain at that level for 12 hr following treatment with BG or dBG. However, in a profile similar to BG, the rate of repletion of MGMT activity following dBG treatment increased with repeated washing. Washed cells recovered approximately 60 % of control activity by 12 hr. These data suggested that dBG similar to BG can be readily washed out of cells.

When cells were treated with 0.1-1.0 mM dBG for 2 hr prior to BCNU administration without a wash, BCNU cytotoxicity increased in a dose dependent manner (Figure

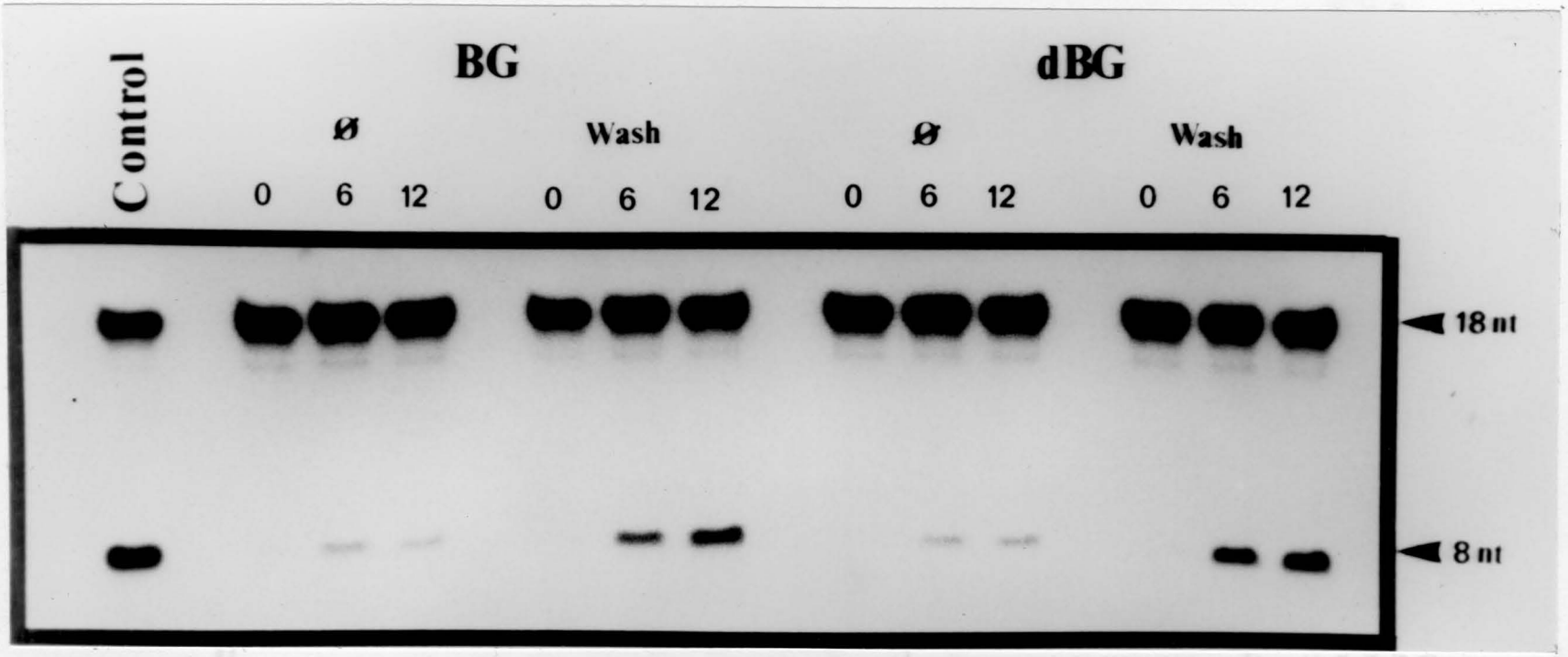


Figure 4.3:

Comparative repletion of MGMT activity in HT-29 cells treated with BG and dBG.

IC_{100} doses of BG or dBG (10μM and 100μM, respectively) were administered to cells for 3 hr and cells were harvested at the indicated times after drug treatment. O^6-methylguanine containing probe was incubated with 10 μg of total cellular protein from untreated (lane 1), BG treated and unwashed (lanes 2-4), BG treated and washed (lanes 5-7), dBG treated and unwashed (lanes 8-10), dBG treated and washed (lanes 11-13) HT-29 cells. Extent of Pvu II cleavage of the 18 nucleotide fragment to an 8 nucleotide fragment is directly proportional to MGMT activity. The wash protocol is described in the "materials and methods".

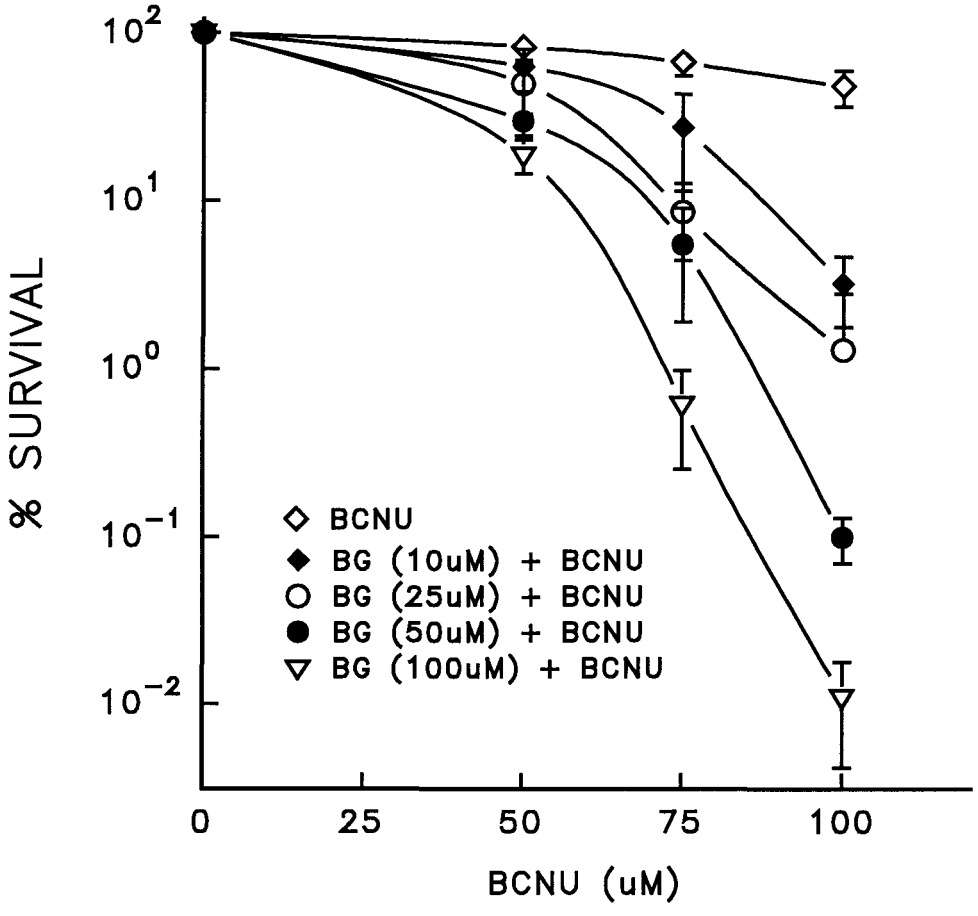


Figure 4.4:

Potentiation of BCNU cytotoxicity by dBG

Cells were pretreated with dBG (0.1-1.0 mM) for 2 hr prior to a 1 hr exposure to various doses of BCNU. After BCNU treatment, drug containing medium was aspirated, replaced with an equal volume of fresh medium, and colony formation was assessed after 10 days. Points, mean and SD of survival values measured in 3 or more replicate flasks.

4.4). This potentiation profile of BCNU cytotoxicity by dBG was extremely similar to BG (10-100 μM) mediated enhancement described in the previous chapter. This observation was consistent with the 10 fold lower affinity of dBG than BG for MGMT. In order to enhance BCNU cytotoxicity comparable to that produced by BG, 10 fold greater doses of dBG were required. Measurement of the repletion kinetics of MGMT activity following dBG (0.1-1.0 mM) treatment (without a wash) revealed a modest dose and time dependent inhibition of MGMT activity (Figure 4.5). The 1.0 mM dose of dBG produced sustained inhibition of MGMT activity for 24 hr and the greatest BCNU cytotoxicity. These data suggested that extended depletion of MGMT following dBG treatment is also important in maximizing BCNU cytotoxicity. However, the activity measured in this experiment was extremely small. Therefore, this hypothesis was further tested by using dBG in a series of MGMT repletion and BCNU potentiation experiments with washes. Figure 4.6 illustrates the recovery of MGMT activity following 1.0 mM dBG alone and in combination with STZ with and without washes. dBG alone without a wash completely inactivated MGMT for 24 hr. Upon repeated washing, the rate of recovery of MGMT activity increased dramatically. As previously shown, the repletion of MGMT activity following STZ was unaffected by washes. The combination of dBG+STZ

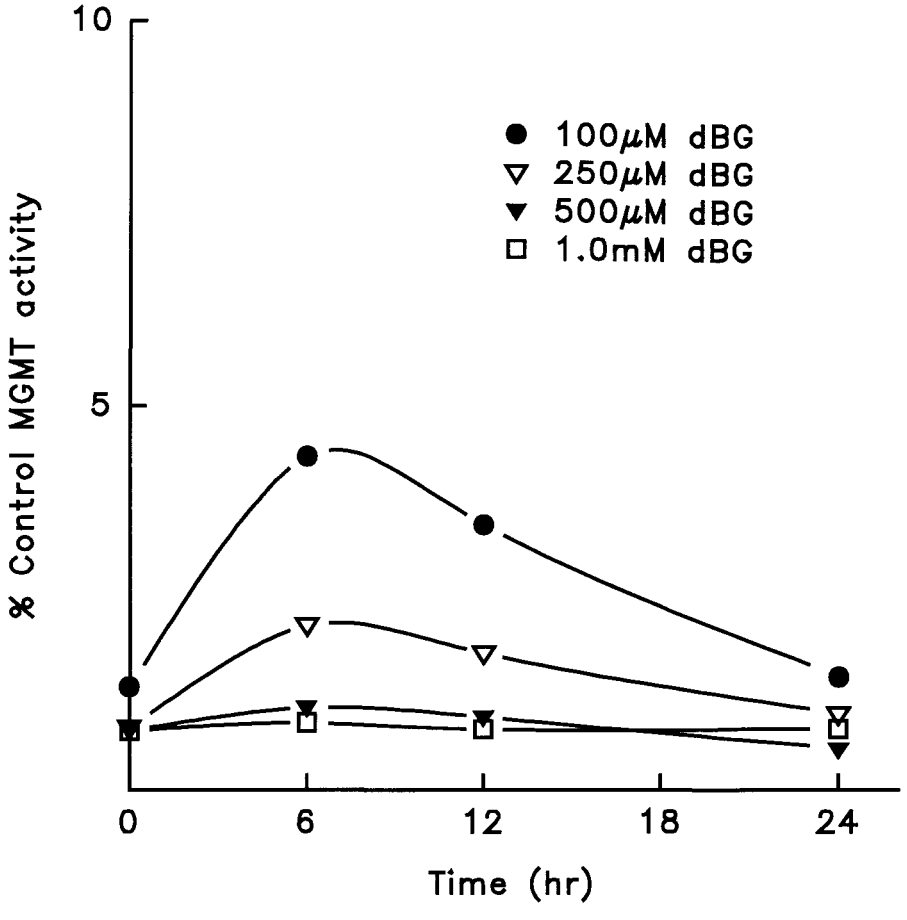


Figure 4.5:

MGMT activity in HT-29 cells following treatment with dBG (100-1000 μM).

Cells were treated with BG (100-1000 μM) for 3 hr, replaced with fresh medium, and harvested at 0, 3, 6, 12, and 24 hr after drug treatment. These data are from a single experiment.

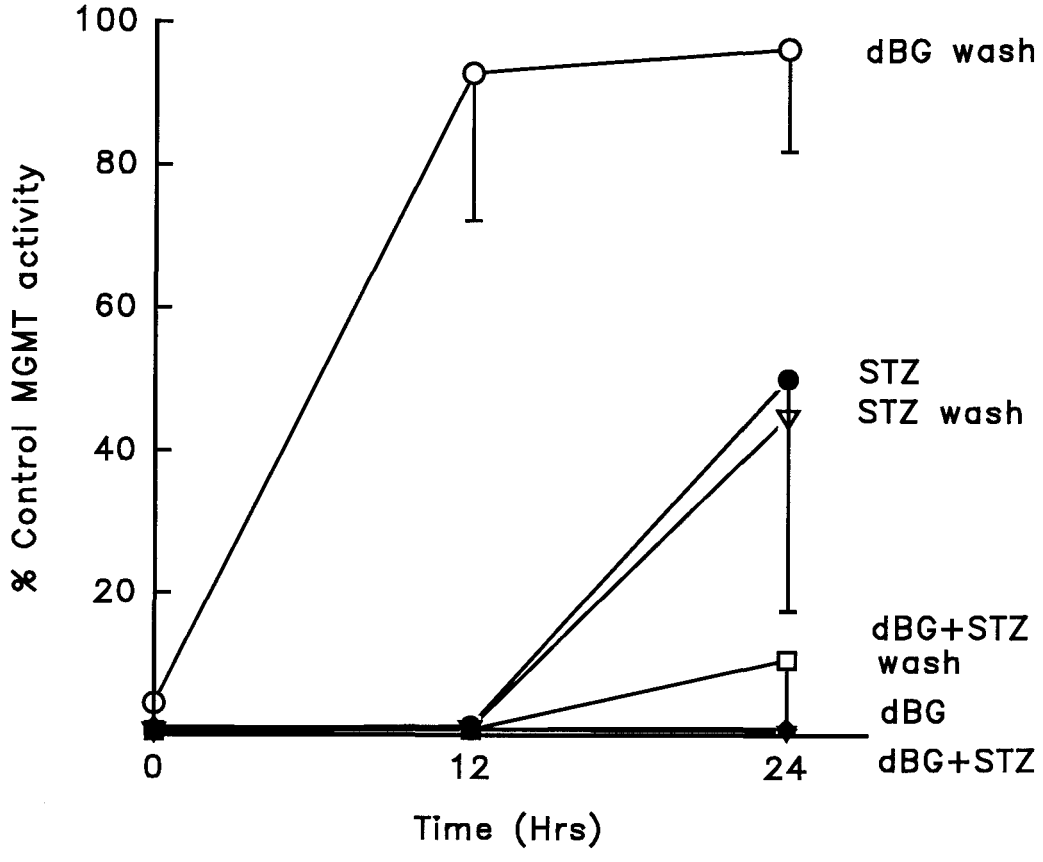


Figure 4.6:

Differential recovery of MGMT activity following dBG, STZ, and the combination of dBG and STZ.

HT-29 cells were administered dBG (1.0 mM) for 3 hr, STZ (2.5 mM) for 2 hr or the combination. Following drug treatment, media was removed, replaced with fresh media or repeatedly washed as described in the "Materials and Methods". Points represent the mean +/- standard deviation of two independent experiments.

produced sustained inhibition of MGMT activity essentially for 24 hr, irrespective of washes. Examination of the potentiation of BCNU cytotoxicity by dBG with or without washes revealed the pattern of reversal of BCNU resistance was very similar to that seen with BG (Figure 4.7). The combination of dBG (1.0 mM) + BCNU (100 μM) without a wash produced approximately 3 logs of synergistic cell kill. Repeated washing of cells drastically increased the recovery rate of MGMT activity and decreased BCNU cytotoxicity. When this recovery was prevented for 24 hr by the addition of STZ, the combination produced three to four logs of synergistic cytotoxicity at a 100 μM dose of BCNU. These data further demonstrated the importance of prolonged depletion of MGMT activity in CENU resistance modulation.

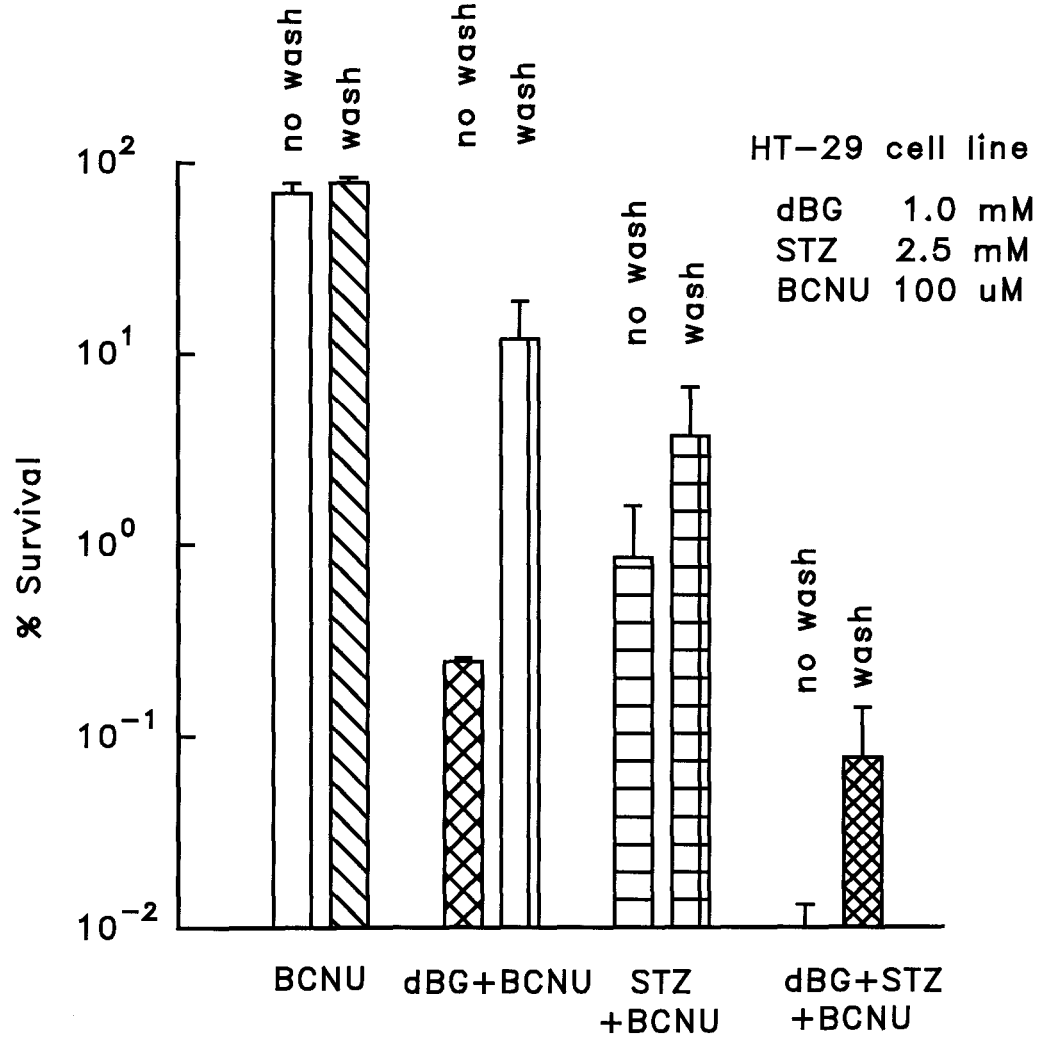


Figure 4.7:

Differential potentiation of BCNU cytotoxicity by dBG, STZ or the combination of dBG+STZ with or without washes.

The protocol utilized is identical to that described in figure 4.2. The points represent the mean +/- SD of three independent experiments.

Discussion

The data presented within this chapter further confirm that extended depletion of MGMT following BG, dBG, and STZ was required to maximize BCNU in vitro. The data showed that pretreatment regimens producing the most extended depletion of MGMT activity for 24 hr produced the greatest enhancement of BCNU cytotoxicity. The recovery rate of MGMT activity following dBG or BG was modulated by repeated washing of cells. BG and dBG, without washing, produced complete depletion of MGMT activity for 24 hr and potentiated BCNU cytotoxicity by about 3 logs. The prolonged depletion resulted presumably from residual BG or dBG remaining within cells. However, washing of cells following BG or dBG administration increased the repletion rate of MGMT; cells recovered to control levels of MGMT by 24 hr. Under these conditions, the enhancement of BCNU cytotoxicity was markedly diminished to less than one log of synergistic cell kill. STZ was combined with BG or dBG to prevent this post-wash recovery of MGMT, and to restore non-wash sensitivity. The post-wash recovery of MGMT activity following BG or dBG administration was inhibited for 24 hr by the addition of STZ. The sequential exposure of BG followed by STZ, or dBG followed by STZ, produced sustained inhibition of MGMT

activity for 24 hr, irrespective of washing. The combination of BG+STZ+BCNU or dBG+STZ+BCNU produced greater than 3 to 4 logs of synergistic cell kill with or without washing.

The repletion profiles of the direct MGMT depleting agents BG and dBG markedly differed from that of STZ after repeated washing of cells. BG and dBG produced sustained inhibition of MGMT for 24 hr without washing, whereas with washing MGMT gradually recovered to control levels by 24 hr. Because BG and dBG are lipophilic molecules, repeated presentation of drug free medium to cells probably facilitated the removal of the compounds via passive diffusion. With the resultant decrease in intracellular concentrations of the free bases, MGMT activity recovered at a faster rate. In contrast, the recovery rate of MGMT following STZ treatment was unaffected by washing. This result is consistent with the mechanism by which STZ depletes MGMT. STZ-induced O^6-methylguanine lesions in DNA facilitate the repair-mediated inactivation of MGMT (Brent, 1986). Washing of cells would not be expected to change the intracellular concentration of this substrate, and therefore not affect the regenerative rate of MGMT.

Dolan et al. (1990a; 1991) suggested that optimal reversal of BCNU resistance required cellular administration of BG pre- and post-BCNU exposure because

the presentation of BG to cells following BCNU facilitated the inactivation of nascent MGMT molecules (Yarosh, et al., 1986; Gerson et al., 1988) and thereby increased BCNU sensitivity. Consistent with this notion, Gerson et al., (1993) suggested that prolonged depletion of MGMT prevented the repair of the stable, cross-link precursor O^6, N^1 ethanoguanine. Prevention of this repair by BG post-BCNU administration could have increased the accumulation of the cytotoxic DNA interstrand cross-links (Gerson et al., 1993).

In BG or dBG treated and washed cells, MGMT activity recovered and presumably repaired any persistent cross-link precursors, and thereby prevented the accumulation of DNA interstrand cross-links. This precursor repair was probably attenuated by the addition of STZ to BG, or dBG. The sequential administration of a guanine analog and STZ probably allowed a greater number of STZ induced O^6-methylguanine lesions in DNA to persist, providing substrates which continued to deplete MGMT activity for 24 hr. This greater extended depletion of MGMT probably allowed an increased number of BCNU cross-links which were responsible for greater cytotoxicity.

The kinetics of BCNU cross-link formation and removal in CENU resistant cell lines depleted of MGMT have not been measured, but the frequency of BCNU induced cross-links did indeed increase when resistant cells were

initially depleted of MGMT by STZ or BG (Futscher et al., 1989; Mitchell et al., 1992). However, these were static determinations made at the estimated times of maximal cross-linking (6 or 14 hr after drug treatment). In an MGMT deficient cell line, BCNU, CCNU, CNU and chlorozotocin induced cross-links continued to form for 6-12 hr after drug removal (Erickson et al., 1980a). The steady state level of cross-links beyond 12 hr could be due to continual formation of interstrand cross-links and repair by perhaps an excision repair mechanism (Swinnen et al., 1991). The potential for continuous cross-link formation was suggested by the remarkable stability of the cross-link precursor O^6, N^1 ethanoguanine; this intermediate was stable in DNA for greater than 8 hr after drug removal (Brent et al., 1987). Extended depletion of MGMT might be required to maximize DNA interstrand cross-links and cytotoxicity in CENU resistant cells because MGMT reacted with this precursor, resulted in the formation of an MGMT-DNA cross-link instead of a DNA interstrand cross-link. This hypothesis might be tested by examining the kinetics of cross-link formation under conditions of complete inhibition, partial, and complete recovery of MGMT activity by 24 hr in CENU resistant cells.

If extended depletion of MGMT was the sole determinant for maximizing BCNU cytotoxicity, then all regimens which

provided complete inhibition of MGMT for 24 hr should have produced comparable cytotoxicities. However, BG and BG+STZ completely inhibited MGMT for 24 hr, but the combination of BG+STZ+BCNU produced at least 1 log greater cytotoxicity than BG+BCNU in an unwashed situation. Likewise, the combination of dBG+STZ potentiated BCNU cytotoxicity greater than dBG alone. The greater cytotoxicity produced by the addition of STZ to BG+BCNU or dBG+BCNU regimen may be attributed to the cytotoxic effects of STZ induced O^6-methylguanine lesions. Day et al (1987) proposed that the O^6-methylguanine lesion could trigger lethality by activating a type of mismatch repair acting on the distortion in DNA created by the inappropriate base pairing of O^6-methylguanine : cytosine. Once this mismatch is detected, several bases in the undamaged strand of DNA are incised and resynthesized (Day et al., 1987). The infidelity of the repair polymerase could generate another mismatch across the O^6-methylguanine lesion (Day et al., 1987). The creation of another mismatch may result in futile cycle of mismatch repair, depleting nucleotide pools, and resulting in the cytostatic effects of methylating agents (Day et al., 1987; Kat et al., 1993).

Kaina et al. (1991) demonstrated that the transfection of the MGMT or *ada* complementary DNAs protected CHO cells from the mutagenic, clastogenic and cytotoxic effects of

MNNG, HeCNU, and the STZ parent compound MNU. These data indicate that O^6-methylguanine is indeed a cytotoxic lesion and MGMT plays a pivotal role in preventing the toxic effects mediated by this lesion. The following remarks are offered to explain the greater cytotoxicity induced by the three-drug combinations (BG+STZ+BCNU and dBG+STZ+BCNU) than any two drug regimen. When MGMT was initially depleted with BG or dBG, a greater number of STZ induced O^6-methylguanine lesions would be expected to form, act in concert with the toxic lesions produced by BCNU, and thereby produce a greater potentiation of BCNU cytotoxicity. However, the cytotoxicities of the three-drug combinations were normalized to cytotoxicities induced by the combination of BG+STZ or dBG+STZ. Whatever intrinsic lethality offered by the addition of STZ would have been accounted for in the normalization process. One may speculate that the cytostatic response mediated by the O^6-methylguanine lesion was greater in the presence of BCNU induced DNA damage than in its absence. If this speculation is correct, the three-drug combination would be expected to produce greater cytotoxicity than the non-STZ containing regimens. Further experiments are clearly required to resolve this issue.

Because the combined administration of the direct MGMT depleting agents, dBG or BG, and the indirect depleting

agent, STZ, sensitized HT-29 cells to BCNU cytotoxicity to a greater degree than modulation with a single agent, the combined clinical testing of resistance modulators with different modes of action might be justified. In chapter three, it was observed that the combination of BG+STZ at 10 fold lower doses than used in this study, effectively sensitized BCNU resistant cells. A clinical regimen utilizing a low dose of BG followed by a low dose STZ might decrease the clinical toxicities observed in the recent STZ+BCNU clinical trials (Micetich et al., 1992). This protocol may produce a more efficient depletion of MGMT than the use of BG or STZ alone. If ample plasma concentrations of BG cannot be achieved to produce sustained inhibition in tumors exhibiting high levels of MGMT due to the limited solubility of the compound, then repeated administration of BG, or the addition of STZ to a BG+BCNU regimen might be necessary. The addition of STZ to a BG+BCNU regimen could result in extended depletion of MGMT, and the STZ-induced DNA damage could increase the tumor cell lesion burden, thereby increasing the possibility of anti-tumor responses. In the following chapter, the enhancement of BCNU cytotoxicity by the combination of BG+STZ is further tested in an <u>in vivo</u> anti-tumor model in order to determine the potential clinical utility of the sequential administration of BG, STZ, and BCNU.

CHAPTER V

ANTI-NEOPLASTIC ACTIVITY OF SEQUENCED ADMINISTRATION OF BG, STZ, AND BCNU *IN VITRO* AND *IN VIVO*

In the previous chapters, the combined administration of BG+STZ produced an extended depletion of MGMT for 24 hr and a greater enhancement of BCNU cytotoxicity than either BG or STZ. Based on these *in vitro* observations, the clinical testing of this combination was recommended. In order to further justify this recommendation, the effectiveness of extended depletion of MGMT for 24 hr on the enhancement of BCNU anti-tumor activity was examined *in vivo* with the combination of BG and STZ. The experiments presented in this chapter used the resistant human glioblastoma SF767 cells grown in culture, and as subcutaneous xenografts in nude mice. The feasibility of the potential clinical use of the three-combination was tested *in vivo* with the subcutaneous xenograft model because this anti-tumor model more reliably predicts the clinical utility of novel chemotherapeutic regimens than *in vitro* cytotoxicity assays.

SF767 astrocytoma cell line was selected on basis of

the rationale developed by Mitchell et al. (1992). These investigators suggested that, because rodent tissue generally contained lower MGMT activity than their human equivalents, the use of a human xenograft with comparable levels of MGMT to host tissues may be more predictive of the clinical effectiveness of a potential chemotherapeutic regimen. Therefore, the SF767 cell line, which contains approximately equivalent MGMT activity to mouse liver (Mitchell et al., 1992; Figure 5.5), was used for the *in vivo* testing of the combination of BG+STZ+BCNU.

Furthermore, the growth rate of SF767 xenografts was significantly inhibited by i.p. administration of BG (80 mg/kg) followed by BCNU (20 mg/kg) without appreciable animal toxicity (i.e. in terms of animal death and nadir in weight loss) (Mitchell et al., 1992). STZ was added to this treatment protocol in order to examine whether the combination of BG+STZ would produce a more extended depletion of MGMT activity and a greater enhancement of BCNU anti-tumor activity than that obtained with BG in cultured SF767 cells and xenografts.

Materials and Methods

Colony Formation Assay

The human glioma cell line, SF767, was kindly provided by The Brain Tumor Research Center, University of California at San Francisco. SF767 cells were cultured in Eagle's minimum essential medium, supplemented with 10% bovine calf serum, 62.5 mM HEPES buffer, 1 mM glutamine, 1 mM sodium pyruvate, 1 X vitamin B12, 1 X non-essential amino acids, and 0.05 mg/ml gentamicin. Cells were maintained in log phase at 37°C in 95% air, 5% CO_2 atmosphere. The cytotoxicity assay used in this chapter was identical to that described in chapter 3.

Animal studies

Female NIH Swiss nude mice and NIH Swiss normal mice were purchased from Frederick Cancer Research and Development Center (Frederick, MD). Animals were housed in a sterile environment and provided with food and water *ad libitum*. The nude mice were subcutaneously injected in the flank region with 7-10 x 10^6 SF767 cells. In tumor growth studies, animals were randomized and treated with various regimens when tumor volumes reached 30-112 mm^3. Animal weights and tumor volumes were measured twice per week. Additionally, animals were euthanized when tumor

exceeded 1500 mm^3. Tumor volumes were calculated using the formula: Length X $Width^2$ X 0.53. In the MGMT repletion studies, tumor volumes ranged from 200 to 400 mm^3 at the time of treatment. BG, STZ, and BCNU were dissolved in 10% cremophor EL, normal saline, and 10% ethanol/90% normal saline, respectively. At various times following drug exposure, mice were euthanized via cervical dislocation. The tumors and organs were dissected, quickly frozen in liquid nitrogen, and stored at -80°C for subsequent MGMT analysis.

Measurement of MGMT activity

The preparation of MGMT extracts and measurement of MGMT activity from cultured cells have been previously described in chapters four and five. In preparing MGMT extracts from SF767 xenografts and organs, the dissected tumors and organs which had been frozen at -80°C were thawed and homogenized for 30 sec in approximately 3 ml/g wet tissue weight of homogenizing buffer containing 50 mM Tris, 5 mM DTT, 0.1 mM EDTA, pH 8 with an OMNI International 1000 tissue homogenizer. The homogenate was centrifuged for 5 min at 12,000X g at 4°C. The cell pellet was reconstituted to approximately 1 ml/g of original wet tissue weight, homogenized for 15 sec and sonicated for an additional 30 sec. Both supernatants were combined and centrifuged at 12,000 X g at 4°C for 30

min. Total cellular protein concentration of the supernatant was quantitated utilizing the Bradford micro protein assay (Bio Rad, Richmond, CA.). To measure MGMT activity, a ^{32}P-labeled 18 base pair oligomer containing an O^6-methylguanine lesion in the Pvu II restriction site was incubated with 10μg or 25μg of whole cell sonicate protein in the homogenizing buffer for 2 hr. After the incubation, the protein was digested with 40μg of proteinase K in the presence of 1% sodium dodecyl sulfate for 1 hr at 45°C. The DNA was purified using a single PCI extraction and ethanol precipitation. The DNA was subsequently digested with 10 units of Pvu II, electrophoresed on a 20% denaturing polyacrylamide gel and autoradiographed. Repair of O^6-methylguanine by MGMT allows Pvu II cleavage of the oligomer to an 8 base pair ^{32}P-labeled fragment. The percentage of restriction enzyme cleavage is directly proportional to MGMT activity. The radioactivity was quantitated using the Betagen betascope 603 blot analyzer. % probe cleaved= (CPMs of 8 mer/CPMs of 18 mer + CPMs of 8 mer) x 100. The values of MGMT activity measured after drug treatment was normalized to untreated control values.

Results

Inactivation of MGMT activity and potentiation of BCNU cytotoxicity by the following pretreatment regimens in SF767 cells; BG, STZ, and the combination of BG+STZ

The human astrocytoma cell line SF767 contains appreciable levels of MGMT and is consequently highly resistant to the cytotoxic effects of CENUs (Dolan et al., 1991). The repletion kinetics of MGMT activity following treatment with BG (10μM), STZ (0.5mM), and the combination of BG+STZ is illustrated in Figure 5.1. MGMT activity was completely inactivated immediately following BG exposure. Thereafter, cells recovered no greater than 15% of control MGMT activity through 24 hr. Following STZ treatment, MGMT was inactivated by greater than 90% of control activity at 0 hr, and recovered to approximately 60% of control activity by 24 hr. However, sequential administration of BG and STZ produced near complete inactivation of MGMT activity through 24 hr. The combination of BG+STZ produced a greater and a more extended inhibition of MGMT activity than either agent alone.

Figure 5.2 illustrates the potentiation of BCNU cytotoxicity by the various pretreatment regimens. BG, STZ, or the combination of BG+STZ were essentially non

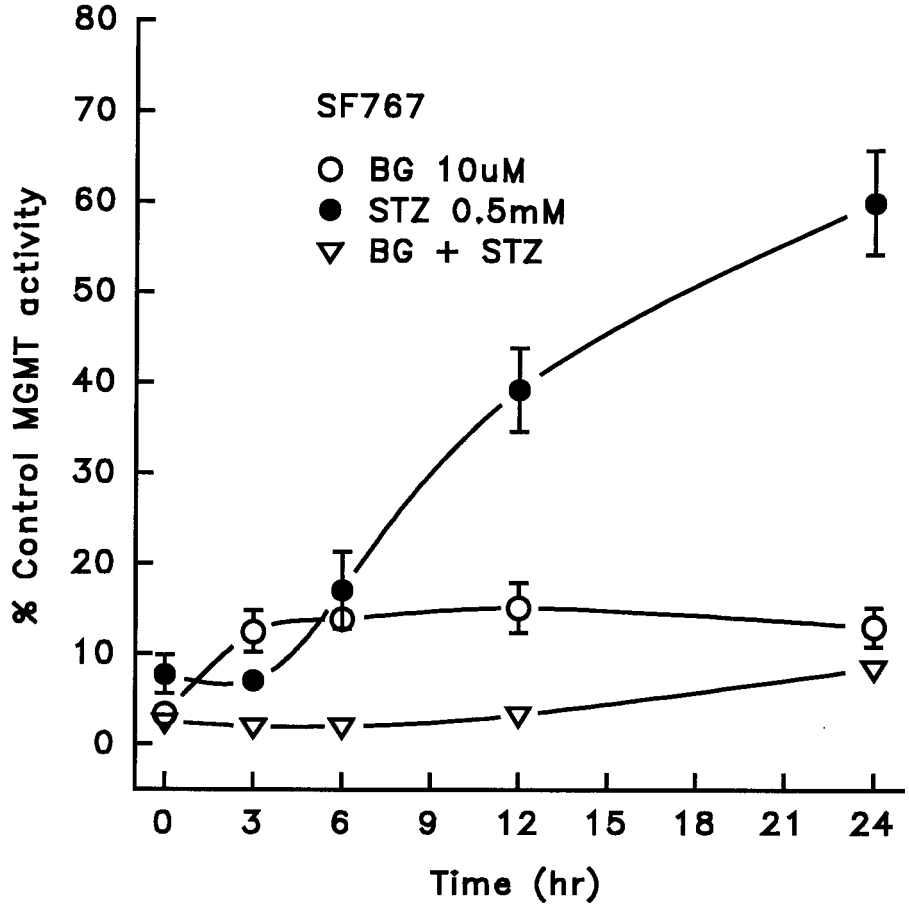


Figure 5.1:

Inactivation and recovery of MGMT activity in SF767 cells <u>in vitro</u>.

SF767 cells were treated with BG (10 μM) for three hours, STZ (0.5 mM) for two hours or the combination. After the three hours the drug containing medium was removed and replaced with fresh medium. The illustrated times refer to the time of cell harvest following drug removal. MGMT activity is expressed as a percentage of activity in untreated SF767 cells. Points represent the mean +/- SD of three independent experiments.

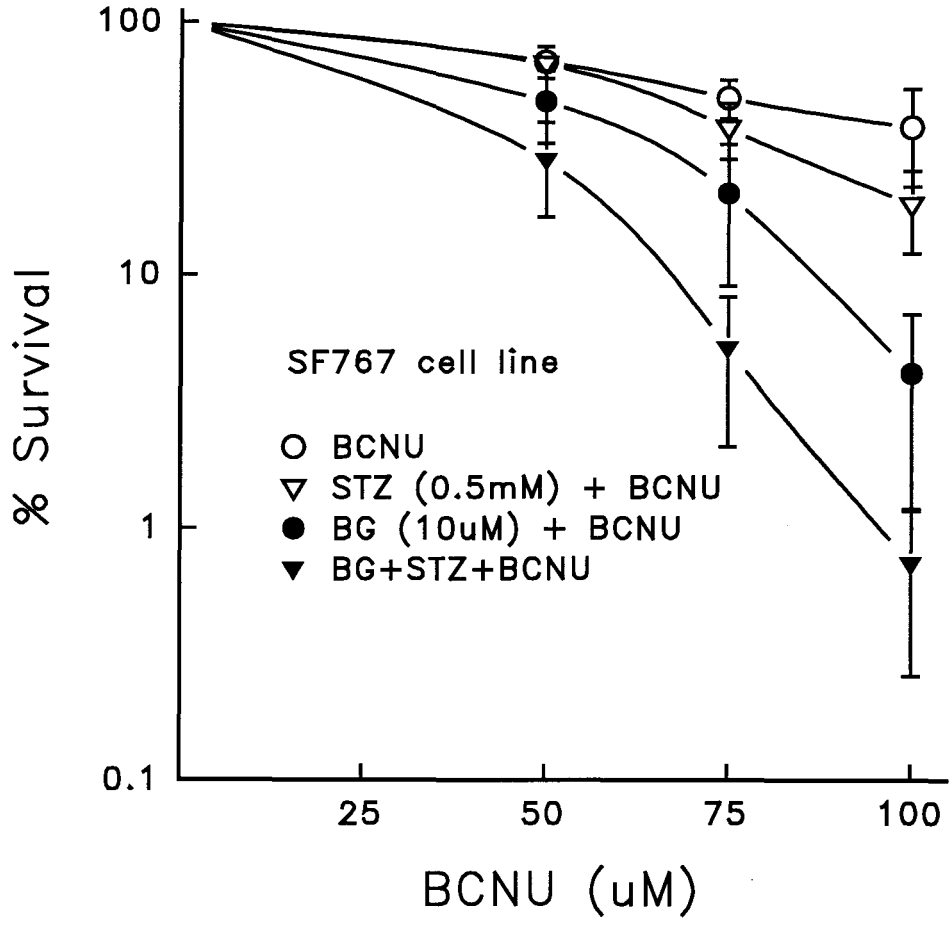


Figure 5.2:

Comparison of the cytotoxicity induced by BG+BCNU, STZ+BCNU, or BG+STZ+BCNU <u>in vitro</u>.

SF767 cells were treated with BCNU (50-100 μM) alone, or STZ (0.50 mM) + BCNU (50-100 μM), or BG (10 μM) + BCNU (50-100 μM), or BG (10 μM) + STZ (0.50 mM) + BCNU (50-100 μM). Cells were exposed to BG for 2 hr and STZ for 1 hr prior to a 1 hr exposure to BCNU. Each data point represents the mean +/- SD of three independent experiments.

Table 5.1:

Survival[a] following the various pretreatment regimens used to enhance BCNU cytotoxicity in SF767 cells

	% Survival
BG (10.0 μM)[b]	96.60 +/- 0.63
STZ (0.50 mM)[b]	87.37 +/- 5.44
BG (10.0 μM) + STZ (0.50 mM)[c]	87.19 +/- 6.24

[a] The survival values reported in this table indicate the percentage of cells surviving +/- the standard deviation after treatment with the various regimens. [b] The cytotoxicity scores for STZ, BG alone are normalized to untreated control values. [c] The combination cytotoxicity values are normalized to scores obtained with BG alone, and therefore reflect the cytotoxicity produced by STZ when combined with BG.

toxic at the doses used in these studies (Table 5.1). Cytotoxicities of BG+BCNU, STZ+BCNU, BG+STZ+BCNU were normalized to BG, STZ, or BG+STZ cytotoxicity. Therefore, the enhancement of BCNU cytotoxicity illustrated in Figure 5.2 by the different pretreatment regimens was synergistic. The combinations of STZ+BCNU, BG+BCNU, BG+STZ+BCNU at 100 μM dose of BCNU potentiated cell killing by approximately 0.5, 1.0 and 2.0 logs, respectively. The enhancement of BCNU cytotoxicity as reflected in the decrease in the IC90 dose of BCNU, is illustrated in Table 5.2. The addition of STZ at a dose of 0.5 mM to the BG+BCNU regimen resulted in a 22.9 % reduction in the IC90 dose of BCNU. The combination of BG+STZ, which produced extended depletion of MGMT produced the greatest sensitization of SF767 cells to BCNU cytotoxicity. These observations were consistent with the determinations made in HT-29 cells in that the combination of BG+STZ produced a more extended depletion of MGMT and a greater enhancement of BCNU cytotoxicity than BG or STZ alone. These data demonstrate that the requirement of extended depletion of MGMT in maximizing BCNU cytotoxicity is not restricted to HT-29 cells.

Table 5.2:

The IC90 doses[a] of BCNU when SF767 cells were treated with the various pretreatment regimens

BCNU alone	> 100 μM
BCNU + STZ (0.5 mM)	> 100 μM
BCNU + BG (10.0 μM)	87.5 μM
BCNU + BG (10.0 μM) + STZ (0.5 mM)	67.7 μM

[a] The concentration of BCNU required to reduce survival of the seeded cells by 90%. The values depicted in this table were extrapolated from the data illustrated in figure 5.2.

Effects of the combination of BG+STZ on the inactivation of MGMT activity and the enhancement of BCNU anti-tumor activity in vivo.

The following series of experiments examined whether the addition of STZ to the BG+BCNU regimen would produce extended depletion of MGMT activity for 24 hr and greater inhibition of tumor growth, or produce tumor regression when compared to the combination of BG+BCNU alone. Preliminary acute toxicity studies using female NIH Swiss regular mice were performed to determine the maximally tolerated dose of STZ in the three drug combination of BG+STZ+BCNU (Table 5.3). Animals were administered the combinations of BG+BCNU or BG+STZ+BCNU i.p. The dose BG was fixed at 80 mg/kg. Animal were treated with BG followed by BCNU at 15 or 20 mg/kg. Alternatively, animals were injected with BG followed by STZ (25-200 mg/kg) followed by the BCNU. The maximally tolerated doses of the three drug combination were inferred to be BG at 80 mg/kg, STZ at 100 mg/kg, and BCNU at 15 mg/kg because these doses produced minimal weight loss and no animal deaths, and because any further increases in the dose of STZ, and/or BCNU resulted in animal deaths. Therefore, the 80 mg/kg dose of BG and the 100 mg/kg dose of STZ were used in MGMT repletion experiments and BCNU anti-tumor experiments in female nude mice bearing SF767 xenografts. Animals were injected with BG, STZ, or the

Table 5.3:

Toxicities of BG+BCNU and BG+STZ+BCNU combinations in normal NIH swiss female mice

Mice were sequentially administered BG, STZ, and/or BCNU i.p. at one hour intervals. Animal weights were measured twice weekly for 28 days.

Combination (mg/kg)	Mean maximum wt loss (%)	# of animal deaths[a]
BG (80)+ BCNU (15)	7.50	0/4
BG (80)+ BCNU (15)+ STZ (25)	13.0	1/4
BG (80)+ BCNU (15)+ STZ (50)	11.0	0/4
BG (80)+ BCNU (15)+ STZ (100)	5.90	0/4
BG (80)+ BCNU (15)+ STZ (200)	10.5	4/12[b]
BG (80)+ BCNU (20)	15.6	2/4
BG (80)+ BCNU (20)+ STZ (25)	13.4	1/4
BG (80)+ BCNU (20)+ STZ (50)	18.1	2/4
BG (80)+ BCNU (20)+ STZ (100)	22.8	3/4

[a]Number of animal deaths/number of animals treated. These data reflected the number of spontaneous deaths and the number of animals sacrificed following 20-25% reduction in total body weight.

[b]Survival through 14 days.

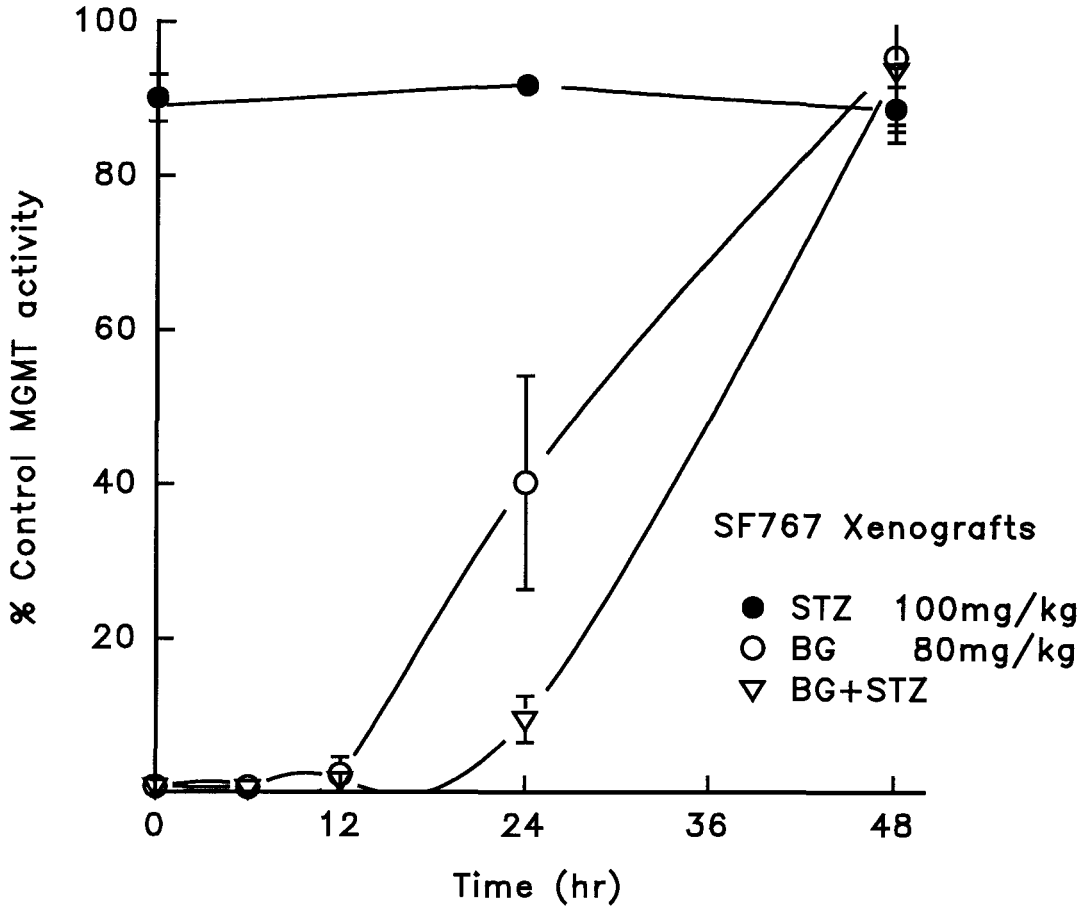


Figure 5.3:

Repletion of MGMT activity in SF767 xenografts following treatment with BG, STZ, or BG+STZ.

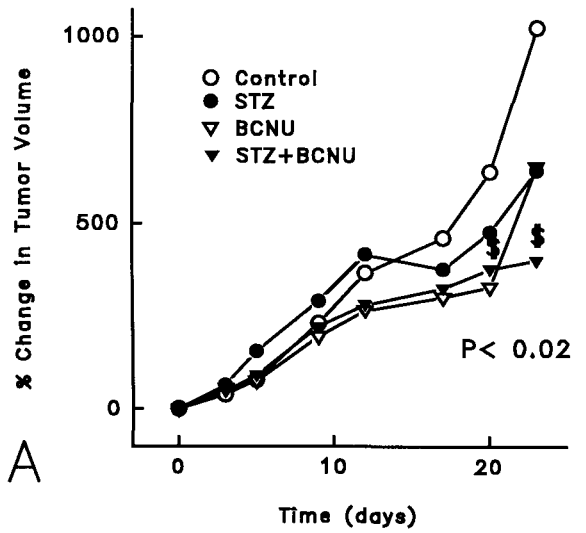
Mice carrying SF767 xenografts were administered 80 mg/kg BG one hour prior to the administration of 100 mg/kg STZ i.p. Animals were sacrificed at the indicated times. The 0 hr time point refers to 3 and 2 hr following BG and STZ treatment, respectively. MGMT activity was expressed as a percentage of activity measured in untreated xenografts. Measurements of STZ alone at 0, 24, and 48 hr, BG and BG+STZ at the 0 and 6 hr time points represent the mean MGMT activity of two animals. The remaining data points represent the mean and SD of three animals.

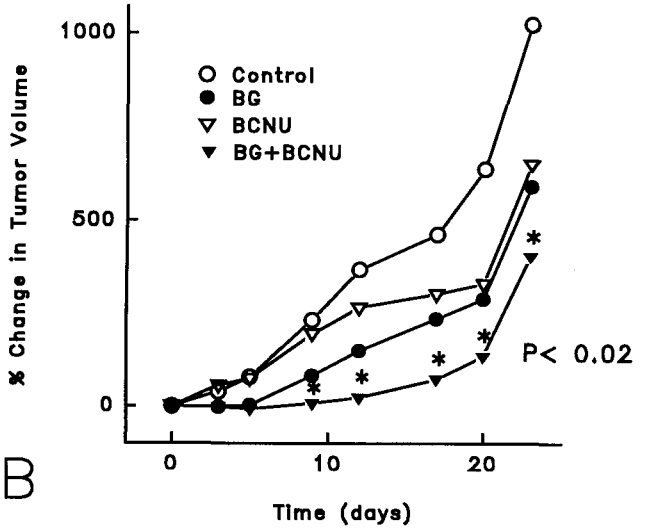
combination of both agents at one hour intervals and were sacrificed at times illustrated in Figure 5.3. Figure 5.3 illustrates the effectiveness of the combination of BG and STZ on the inactivation and recovery of MGMT activity in SF767 xenografts. As a single agent, STZ (100 mg/kg) did not appreciably affect MGMT activity in the xenografts whereas, complete inhibition of MGMT activity for 12 hr wasobserved following BG (80 mg/kg) administration. Thereafter, MGMT recovered to approximately 40% of control levels by 24 hr. Sequenced administration of BG (80 mg/kg) and STZ (100 mg/kg) produced a slower recovery rate of MGMT activity than BG alone. The combination produced near complete inhibition of MGMT activity for 24 hr. Thereafter, the tumors recovered control levels of MGMT activity by 48 hr. These data were consistent with the <u>in vitro</u> observations in that the combination of BG+STZ produced extended depletion of MGMT for nearly 24 hr. These data further suggest that the sequential administration of BG and STZ probably allows a greater number of STZ induced O^6-methylguanine lesions in DNA to persist and deplete MGMT for 24 hr in the xenografts. Nude mice bearing SF767 xenografts were sequentially administered the maximally tolerated doses of BG, STZ, and BCNU to test whether this three-drug combination would provide greater anti-tumor activity than BG+BCNU. Figure 5.4 illustrates the anti-

Figure 5.4:

Tumor growth inhibition assay utilizing subcutaneously implanted SF767 xenografts.

Nude mice carry the xenografts were injected i.p. with BG (80 mg/kg), STZ (100 mg/kg), and/or BCNU (15 mg/kg) at one hour intervals. Each data point represents the mean percent change in tumor volumes of 5-9 animals. The individual tumor volumes throughout the study were normalized to pretreatment values for each individual tumor. Panel A, B, and C depict the anti-tumor activity in animals receiving the combination of STZ+BCNU, BG+BCNU, BG+STZ+BCNU, respectively. Within panel D, the tumor growth curves of untreated, BCNU, BG+BCNU, STZ+BCNU, BG+STZ+BCNU treated animals are reproduced for comparison. *Statistical analysis*, the data were analyzed with an multivariate ANOVA using repeated measures with a post-hoc test of single degree of freedom contrast. The analysis was performed with the SYSTAT™ statistical package.


1000
500
0
% Change in Tumor Volume
Control
STZ
BCNU
STZ+BCNU
P< 0.02
0
10
20
Time (days)
A


1000
500
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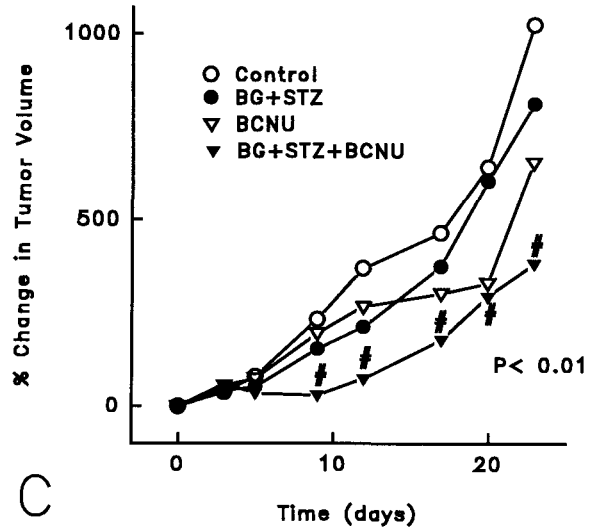
% Change in Tumor Volume
Control
BG
BCNU
BG+BCNU
P< 0.02
0
10
20
Time (days)
B


1000
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% Change in Tumor Volume
Control
BG+STZ
BCNU
BG+STZ+BCNU
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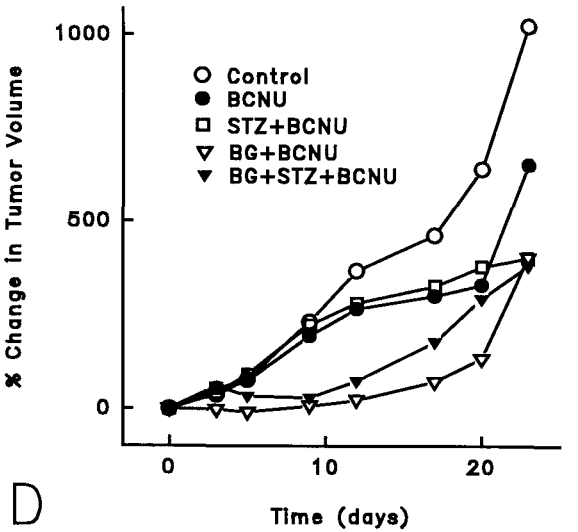
1000
500
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% Change in Tumor Volume
Control
BCNU
STZ+BCNU
BG+BCNU
BG+STZ+BCNU
0
10
20
Time (days)
D

tumor activity of all combinations of BG, STZ, and BCNU. Panels A, B, and C depict the results of tumor growth inhibition assays in animals receiving STZ+BCNU, BG+BCNU, and BG+STZ+BCNU, respectively. The combination of STZ+BCNU produced significantly greater inhibition of tumor growth on days 20 and 23 ($p < 0.02$) than untreated controls. However, BG which depleted xenograft MGMT for 12 hours produced significant inhibition of tumor growth for 9-23 days relative to the untreated controls ($p < 0.02$). These results are consistent with the observations made by Mitchell et. al (1992) in that the combination of BG+BCNU produced significant inhibition of SF767 xenograft growth for greater than 20 days. Similarly, the three drug combination of BG+STZ+BCNU also significantly inhibited tumor growth for 23 days as compared to the untreated controls ($p < 0.01$). However, tumor regressions, defined as reductions in tumor volume over two consecutive measurements, were not observed in any of the tumors treated with the various combinations. In panel D, the tumor growth rates from untreated animals and those treated with BCNU, STZ+BCNU, BG+BCNU and BG+STZ+BCNU are reproduced for comparison. The combination of BG+BCNU produced significantly greater anti-tumor activity on days 9, 12, 17, and 20 ($p < 0.03$) than STZ+BCNU. Similarly, the combination of BG+STZ+BCNU produced greater anti-tumor activity than STZ+BCNU on

Table 5.4:

Toxicities of STZ, BCNU, or the combinations of BG+STZ, BG+BCNU, STZ+BCNU, BG+STZ+BCNU

Female nude mice[a] bearing SF767 xenografts were sequentially administered BG, STZ, and/or BCNU i.p. at 1 hr intervals. Animal weights were measured twice weekly for 23 days.

Combination (mg/kg)	Mean maximum weight loss (%)	# of animal deaths[b]
STZ (100)	<1 %	0/7
BCNU (15)	<1 %	0/6
BG (80) + STZ (100)	<1 %	0/7
BG (80) + BCNU (15)	4.8 %	0/8
STZ (100) + BCNU (15)	<1 %	0/7
BG (15) + STZ (100) + BCNU (15)	2.4 %	0/7

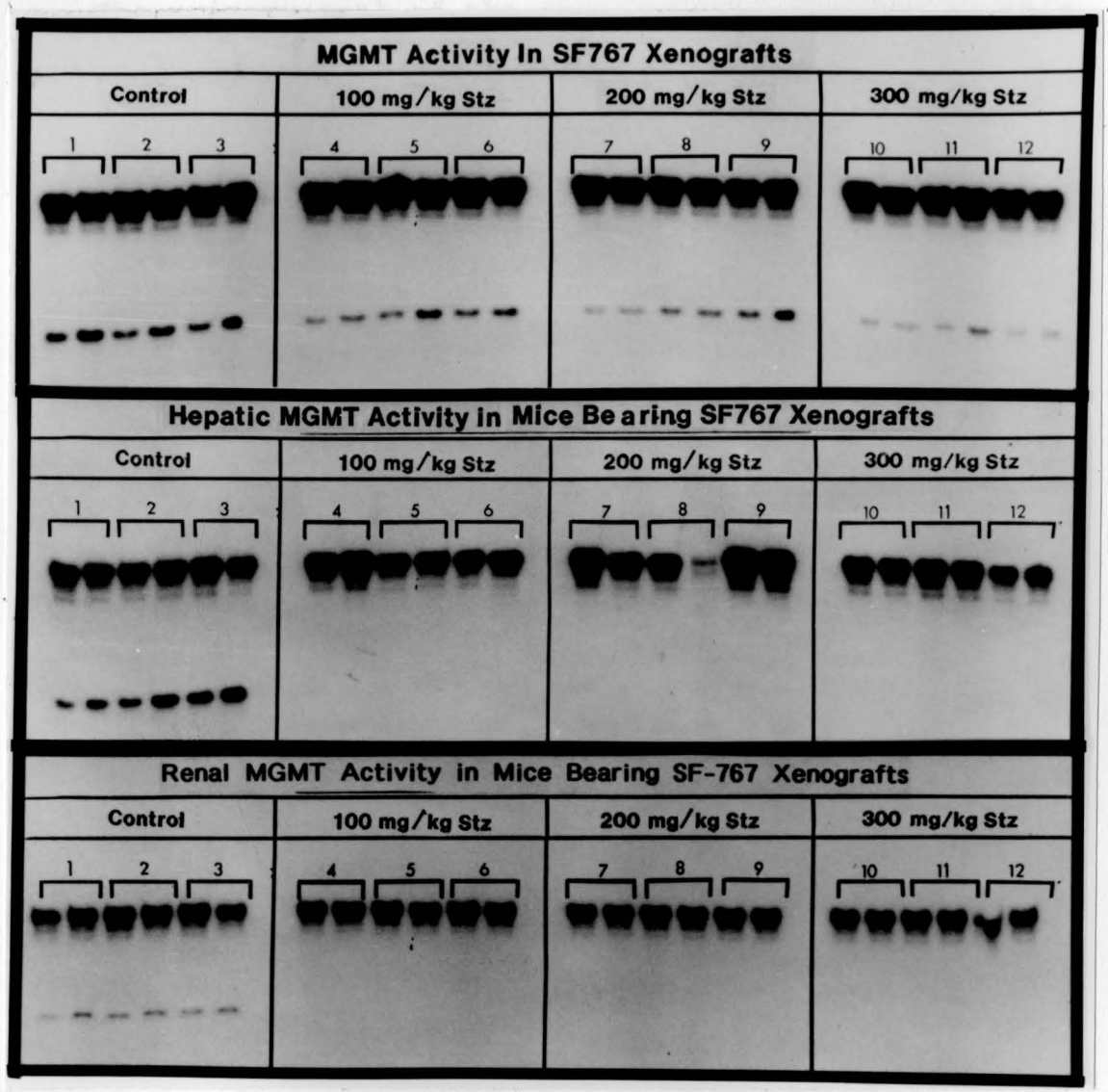
[a] NIH Swiss nude mice
[b] Number of animal deaths/number of animals treated

days 9 and 12 ($p < 0.05$). However, the three drug combination of BG+STZ+BCNU provided no greater anti-tumor activity than BG+BCNU. Additionally, the administration of all combinations of BG, STZ, and BCNU did not appreciably increase nude mouse toxicities (Table 5.4). These data demonstrate the feasibility using the combination of BG and STZ to provide extended depletion of MGMT for 24 hr without exacerbating BCNU toxicities. However, the potential therapeutic benefit offered by the addition of STZ to the BG+BCNU combination could not be demonstrated in the nude mouse subcutaneous anti-tumor model. The doses of STZ in this model were probably limited by mouse toxicities due to low levels of MGMT activity in host tissues. Consistent with this interpretation was that a single dose of 100 mg/kg STZ which was ineffective in depleting xenograft MGMT, nevertheless, produced complete ablation of renal and hepatic MGMT activity (Figure 5.5). These data demonstrate that in the nude mouse subcutaneous anti-tumor model, BG as a single agent can produce complete depletion of MGMT for 12 hr, greater anti-tumor activity than STZ+BCNU, and anti-tumor activity equivalent to the three-drug combination.

Figure 5.5:

The effect of STZ on MGMT activity in SF767 xenografts, kidneys, and livers of NIH swiss nude mice.

Animals were injected i.p. with normal saline or STZ at the dose of 100 mg/kg, 200 mg/kg, and 300 mg/kg. Animals were sacrificed six hours following drug treatment animals were sacrificed. The bracketed doublet lanes represent MGMT activity with in a single tumor or organ where the O^6-methylguanine containing probe was incubated with 10 or 25 μg protein.


MGMT Activity In SF767 Xenografts
Control
100 mg/kg Stz
200 mg/kg Stz
300 mg/kg Stz
1 2 3
4 5 6
7 8 9
10 11 12
Hepatic MGMT Activity in Mice Bearing SF767 Xenografts
Control
100 mg/kg Stz
200 mg/kg Stz
300 mg/kg Stz
1 2 3
4 5 6
7 8 9
10 11 12
Renal MGMT Activity in Mice Bearing SF-767 Xenografts
Control
100 mg/kg Stz
200 mg/kg Stz
300 mg/kg Stz
1 2 3
4 5 6
7 8 9
10 11 12

Discussion

The primary goal of the experiments described in this chapter was to evaluate the effectiveness of the combined use of two MGMT depleting agents, BG and STZ, in reversing BCNU resistance in in vivo. The effects of the combination of BG+STZ on the repletion of MGMT and the enhancement of BCNU cytotoxicity was initially measured in cultured SF767 cells. The sequential administration of BG and STZ sensitized SF767 cells to BCNU cytotoxicity and produced a more prolonged inhibition of MGMT activity than either agent alone. The combined use of BG+STZ depleted MGMT activity for 24 hr and produced approximately 2 logs of synergistic BCNU cell kill. Consistent with these in vitro observations, the combination of BG+STZ also produced a more prolonged inhibition of MGMT activity in SF767 xenografts than either agent alone. The combination produced sustained inactivation of MGMT activity for 24 hr in the xenografts. Both combinations, BG+BCNU and BG+STZ+BCNU, produced significant inhibition of tumor growth for 9 to 23 days after treatment and demonstrated a greater anti-tumor activity than STZ+BCNU.

The use of STZ as a BCNU resistance modulator in vivo appears to be limited. STZ at 100 mg/kg administered i.p. did not deplete MGMT activity in SF767 xenografts

but completely depleted renal and hepatic MGMT activity (Figure 5.5). Furthermore, a 300 mg/kg dose of STZ produced only a 35% reduction in xenograft MGMT (Figure 5.5). Because this particular dose of STZ was close to the acute LD50 dose (360 mg/kg) (Iwasaki et al., 1976), the concentrations required to inhibit neoplastic MGMT activity would be expected to produce unacceptable host toxicity and thereby provide no beneficial change in the therapeutic index of BCNU. Similarly, Friedman et al. (1992) administered 300 mg/kg of STZ to BALB/c mice bearing medulloblastoma and glioblastoma tumors and found that this dose of STZ did not appreciably deplete MGMT activity, nor did it enhance BCNU sensitivity. Moreover, the DNA methylating agents temozolamide and dacarbazine were also ineffective in sensitizing SF767 xenografts to BCNU cytotoxicity (Mitchell and Dolan, 1993). These data suggest that methylating agents should not be used as single agents for CENU resistance modulation. However, STZ might be useful when added to the BG+BCNU regimen. It may be advantageous to use a MGMT depleting agent with intrinsic anti-tumor activity, such as STZ, in combination with BG. In the sequential administration of BG, STZ, and BCNU, BG can deplete MGMT, and thereby allow a greater number of STZ induced O^6-methylguanine lesions to persist and deplete MGMT for extended periods. These additional STZ lesions could act in concert with BCNU

adducts to increase the tumor cell lesion burden and produce a greater clinical anti-tumor activity. This rationale for the use of the three-drug combination was detailed in chapter 4. The O^6-methylguanine lesion has been considered to be more mutagenic than cytotoxic. However, recent observations by Kaina et al. (1991) demonstrated that transfection of MGMT and *ada* complementary DNAs into MGMT deficient cells produced significant protection against methylating agent cytotoxicity, as well as mutagenesis. This cytotoxic potential of STZ induced O^6-methylguanine lesions could be maximized when combined with BG.

The combination of BG+STZ+BCNU produced extended depletion of MGMT and greater cytotoxicity than BG+BCNU or STZ+BCNU in both HT-29 and SF767 cell lines. The pre-treatment regimens which provided sustained inhibition of MGMT for at least 24 hr after BCNU treatment produced the greatest potentiation of BCNU cytotoxicity <u>in vitro</u>. The enhancement of BCNU cytotoxicity in SF767 cells was markedly less than in HT-29 cells. This lower enhancement in SF767 cell line would be expected because the magnitude of enhancement of CENU cytotoxicity is a function of cellular MGMT content (Dolan., 1991; Chen et al., 1993a,b), and because SF767 cells contains a 30 % lower MGMT activity than HT-29 cells.

At the maximally tolerated doses, the combination of

BG+STZ+BCNU produced significant inhibition of tumor growth. However, this three-drug combination provided no greater anti-tumor activity than BG+BCNU. Both regimens produced significant inhibition of tumor growth for 9-23 days and produced minimal host toxicity. These data suggest that the addition of STZ to a BG+BCNU regimen might not be necessary. In the mouse, ample tumor concentrations of BG were reached to inactivate MGMT for 12 hr and this prolonged inhibition appeared to be sufficient in maximizing tumor responses in the xenograft model. These and other data strongly recommend the clinical testing of the combination of BG+BCNU (Dolan et al., 1990a,b; 1991; Mitchell et al., 1992; Gerson et al., 1993; Friedman et al., 1992). However, one may not achieve high enough plasma concentrations of BG to inactivate tumors with high levels of MGMT for a prolonged period due to the limited solubility of the compound. This difficulty could be resolved by the repeatedly administering of BG following BCNU administration, or by using the combination of BG+STZ. Repeated administration of BG provides complete depletion of MGMT activity 24 hr and potentiates BCNU cytotoxicity in cultured cells and tumor xenografts (Gerson et al., 1993). Alternatively, the combined use of BG and STZ could not only provide extended depletion of MGMT, but also the addition of STZ-induced DNA damage could

potentially contribute to clinical anti-tumor activity. The advantages and disadvantages of the combined use of BG and STZ in BCNU resistance modulation is further discussed below.

CHAPTER VI

SUMMARY

The experiments described within this dissertation tested whether extended depletion of MGMT activity following BG, dBG, and/or STZ was required for maximal enhancement of BCNU cytotoxicity in CENU resistant cell lines. The potentiation of BCNU cytotoxicity was examined after complete inhibition, partial or complete recovery of MGMT activity 24 hr following BG, dBG, and STZ treatment.

The pretreatment regimens which provided the greatest potentiation of BCNU cytotoxicity were those exhibiting the most prolonged depletion of MGMT activity. Treatment of HT-29 cells with a 10 μM dose of BG resulted in complete inactivation of MGMT immediately after drug treatment, with a recovery of approximately 15% of control activity in 3-24 hr. This partial recovery of MGMT activity was completely inhibited for 24 hr by combining BG (10 μM) with STZ (1.0 mM), or by increasing the dose of BG to 100 μM. The prolonged depletion of MGMT activity provided by the combination of BG (10 μM) + STZ (1.0 mM) or BG (100 μM) was associated with a greater enhancement of BCNU cytotoxicity than the dose of 10μM

BG. Additionally, the recovery rate of MGMT activity following BG (100 μM) or dBG (1.0 mM) treatment was modulated by repeated washing of cells with complete medium. Subsequent to BG (100 μM) or dBG (1.0 mM) treatment, MGMT activity was completely inactivated for 24 hr. Additionally, this pretreatment regimen enhanced BCNU cytotoxicity by 3 logs. MGMT activity recovered to control levels by 24 hr, and BCNU cell kill was coordinately reduced to less than one log when cells were pretreated with BG or dBG and washed four times with serum containing medium. When this recovery was completely inhibited for 24 hr by combining BG or dBG with a 2.5 mM dose of STZ, BCNU cytotoxicity was potentiated by 4 logs. These data indicate that MGMT should be depleted prior to, and for an extended period following BCNU treatment. The extended depletion of MGMT may allow an increased number of BCNU interstrand cross-links resulting in greater cytotoxicity. Furthermore, the sequential administration of the direct MGMT depleting agents BG or dBG, and STZ probably results in a greater number of STZ-induced O^6-methylguanine lesions which are responsible for the extended depletion of MGMT. The consistently greater enhancement of BCNU cytotoxicity by the combination of BG+STZ or dBG+STZ than by BG alone under conditions of comparable MGMT depletion, suggests that the greater number of STZ lesions act in concert

with BCNU alkylation to produce the greater cytotoxicity *in vitro*.

This rationale for the combined use of BG and STZ in BCNU resistance modulation was tested *in vivo* utilizing the SF767 subcutaneous xenograft anti-tumor model. Consistent with the *in vitro* observations, the combination of BG+STZ produced near complete depletion of MGMT for 24 hr, whereas BG alone allowed a recovery of 40% control MGMT activity by 24 hr. The sequential administration of BG, STZ, and BCNU, at maximally tolerated doses produced no greater anti-tumor activity than the combination of BG+BCNU *in vivo*.

Collectively, these data suggest that clinical chemotherapeutic regimens targeting the inactivation of MGMT, and the reversal of BCNU resistance, should be optimized such that MGMT is depleted prior to, and for an extended period after BCNU treatment. If extended depletion of MGMT cannot be achieved with BG due to solubility limitations in a clinical setting, then this difficulty may be overcome in two ways: i) repeated administrations of BG could provide complete depletion for 24 hr and potentiate BCNU cytotoxicity *in vitro* and *in vivo* (Gerson et al., 1993); ii) alternatively, BG could be combined with STZ. The latter regimen could act as a proverbial double-edged sword. Serial administration of BG and STZ could not only provide

extended depletion of MGMT, but also the enhanced number of O^6-methylguanine lesions could contribute to anti-tumor activity.

If clinical CENU resistance modulation were to require multiple cycles of chemotherapy, the combined use of BG and STZ could also prevent the possible selection of BG resistant tumor cells. A single point mutation within MGMT has been demonstrated to impair the MGMT-mediated repair of BG, whereas the repair of O^6-methylguanine in DNA was unaffected (Crone and Pegg, 1993). The addition of STZ to a BG+BCNU regimen could provide ample levels of O^6- methylguanine in DNA which could inactivate BG resistant MGMT, and thereby facilitate BCNU cell killing.

A potential clinical disadvantage to the BG+STZ+BCNU regimen could be an increased risk of secondary malignancies. MGMT plays a pivotal role in the prevention of methylating agent induced mutagenesis and carcinogenesis (Kaina et al., 1991; Dumenco et al., 1993). Presentation of methylating agent-induced pro-carcinogenic lesions when MGMT is depleted with BG could conceivably increase mutation rate, and thereby increase the possibility of secondary neoplastic transformation.

The combination of BG+BCNU has produced promising results in pre-clinical studies. The clinical testing of this regimen should consider the importance of extended depletion of MGMT in maximizing CENU cytotoxicity. With

the development of treatment modalities which can selectively deliver BG to tumor cells, an increased CENU therapeutic index and spectrum of clinical activity is anticipated.

APPENDIX I

Upon review of the literature concerning the modulation of alkylating agent resistance, Tiecher and Frei (1991) concluded that neoplastic drug resistance is multifactorial and optimal clinical modulation of such resistance should target multiple resistance mechanisms simultaneously. Utilizing this rationale, the effect of combined depletion of MGMT and GSH on BCNU sensitivity in HT-29 cells was examined (Figure A.1). Cells were sequentially administered BSO, BG, and BCNU. Partial and complete depletion of GSH and MGMT, respectively, resulted in a greater enhancement of BCNU cytotoxicity than any two-drug regimen. BG increased BCNU (100 μM) cell killing by approximately 1.5 logs, and the addition of BSO to this regimen provided an additional 0.5 log of cytotoxicity. These data suggest that GSH may indeed be a secondary mechanism of CENU resistance in CENU resistant cells and further development of regimens targeting multiple mechanisms of resistance may be warranted.

Pretreatment of brain tumor cell lines with diflouromethylornithine (DFMO), an inhibitor of the rate limiting enzyme in polyamine synthesis, decreased

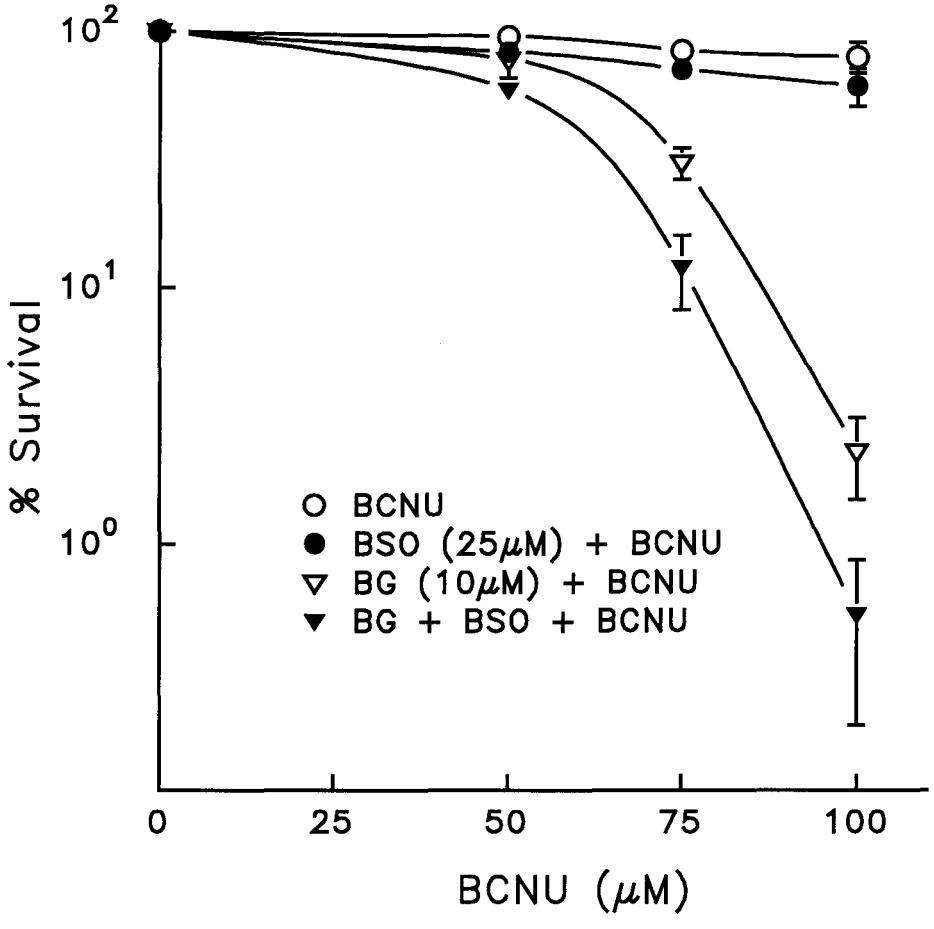


Figure A.1:

The effects of sequential administration of BSO, BG, and BCNU on HT-29 cytotoxicity

Cells were treated with BSO (25 μM) and BG (10 μM) for 24 hr and 2 hr, respectively, prior to a 1 hr exposure to BCNU. BSO at a dose of 25 μM produced approximately 80% reduction in GSH content in HT-29 cells after 24 hr of exposure (Hantel et al., 1991). The 10 μM dose of BG utilized represents the IC_{100} dose for MGMT inhibition (Chapter 3). The combination of BSO+BG was non-cytotoxic (data not shown). The tissue culture and survival assay protocols were described in the material and methods section of chapter 3. The points represent the mean +/- SD of survival values in 3 or more replicate flasks in a single experiment.

spermidine and putrescine levels, and increased BCNU cytotoxicity (Hunget al., 1981; Seidenfeld and Komar, 1985). The decrease in polyamine levels appears to be related to an increased DNA damaging effects of BCNU. The combined depletion of MGMT and ornithine decarboxylase by BG and difluromethylornithine produces greater cytotoxicity than BG+BCNU or DFMO+BCNU (M.E. Dolan, personal communication). Furthermore, the topoisomerase II inhibitor novobiocin markedly increases the antitumor effect of BCNU (Ross, 1985; Eder et al., 1989). This inhibition may prevent the disaggregation of chromosomes in the G_2 and M periods of the cell cycle and concurrently inhibit DNA repair. Therefore, it could be of therapeutic interest to simultaneously deplete MGMT, polyamine levels and inhibit topoisomerase II. Modulation of multiple mechanisms of CENU resistance could coordinately allow a greater DNA damage and a lower DNA repair proficiency. The increased DNA damage or impairment of DNA repair would be expected to increase the clinical antitumor activity of BCNU than depletion of MGMT alone.

Therefore, a multimodulatory approach could also decrease the possibility of acquired cellular resistance to BG. A single point mutation within the MGMT cDNA markedly decreases BG sensitivity, and therefore mediate BG resistance (Crone and Pegg, 1993). Repeated

administration of BG+BCNU could result in the selection of a subpopulation of tumor cells expressing BG resistant MGMT. Concurrent targeting of multiple sites of CENU resistance could result in a greater cell killing and possibly prevent the re-establishment of tumor mass by BG-resistant cells.

Appendix II

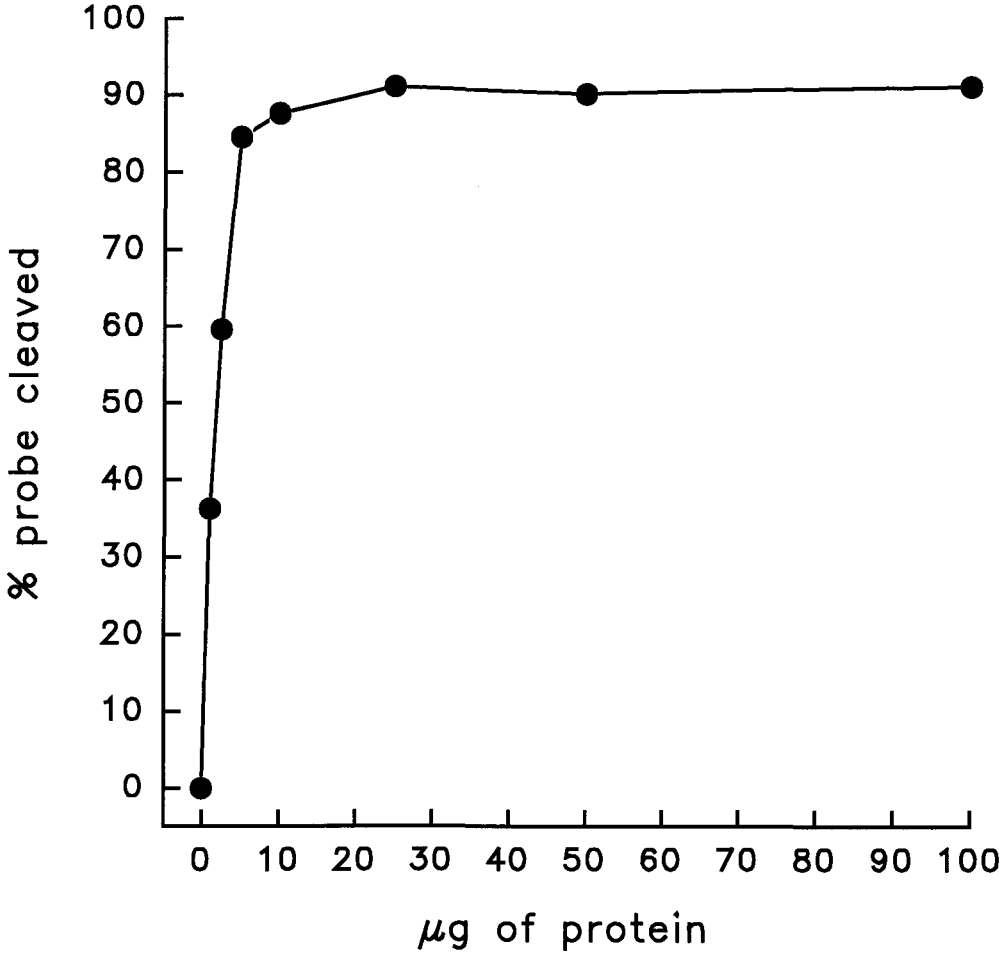


Figure A.2:

The effect of increasing amounts of MGMT containing extract incubated with approximately 0.2 pmoles of O^6-methylguanine containing DNA on the percentage of probe cleaved.

The radiolabelled probe was incubated with 0-100 μg of HT-29 whole cell sonicate for 2 hr at 37°C. The protocol used in this experiment was described in the "Methods and Materials" section of chapter 3.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with the reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in pharmacology.

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