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Laboratory Investigation

Gene expression profile induced by BCNU in human glioma cell lines with differential MGMT expression

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Summary

Chemotherapy with the alkylating agent BCNU (1,3-bis (2-chloroethyl)-1-nitrosourea) is the most commonly used chemotherapeutic agent for gliomas. However, the usefulness of this agent is limited because tumor cell resistance to BCNU is frequently found in clinical brain tumor therapy. The O^6-methylguanine-DNA methyltransferase protein (MGMT) reverses alkylation at the O^6 position of guanine and we have reported the role of MGMT in the response of brain tumors to alkylating agents. However, the different mechanisms underlying the patterns related to MGMT remain unclear. To better understand the molecular mechanism by which BCNU exerts its effect in glioma cell lines according MGMT expression, we used microarray technology to interrogate 3800 known genes and determine the gene expression profiles altered by BCNU treatment. Our results showed that treatment with BCNU alters the expression of a diverse group of genes in a time-dependent manner. A subset of gene changes was found common in both glioma cell lines and other subset is specific of each cell line. After 24 h of BCNU treatment, up-regulation of transcription factors involved in the nucleation of both RNA polymerase II and III transcription initiation complexes was reported. Interestingly, BCNU promoted the expression of actin-dependent regulators of chromatin. Similar effects were found with higher BCNU doses in MGMT+ cell line showing a similar mechanism that in MGMT-deficient cell with standard doses. Our data suggest that human glioma cell lines treated with BCNU, independently of MGMT expression, show changes in the expression of cell cycle and survival-related genes interfering the transcription mechanisms and the chromatin regulation.

Introduction

Human malignant glioma is mostly a fatal disease. Surgical resection of the primary tumors followed by irradiation and chemotherapy with the alkylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine, BCNU), the most commonly used chemotherapeutic agent for gliomas, can increase the survival but the tumor typically recurs [1,2]. Only 20–30% of these patients benefit from adjuvant chemotherapy. Why almost 3 out of 4 patients fail chemotherapy, and why the others respond, still remains unclear. Therefore, it is a major task for the future to identify in advance those patients who will respond to chemotherapy, and develop new therapeutic strategies that will increase the overall frequency of response.

Several mechanisms of resistance are involved for BCNU. One of the best-studied mechanisms is the DNA repair protein MGMT (O^6-methylguanine-DNA methyltransferase). O^6-Alkylguanine is only one of many products formed in DNA by the reaction of alkylating agents but it is of the major importance in both the initiation of mutations and cytotoxic actions of these agents. MGMT repairs DNA damage by removing alkyl groups from the O^6 position of guanine, a critical site of alkylation by the nitrosoureas. In some cells, resistance to BCNU and other alkylating agents can be mediated by MGMT, and depletion of MGMT activity by O^6-benzylguanine has been shown to reverse resistance [3,4]. Moreover, the level of expression of MGMT is closely related to the cytotoxic activity of alkylating agents [5] , and several data suggest that tumors with a low MGMT level are more sensitive to alkylating chemotherapy [6–8]. In this sense, we have previously reported the role of MGMT in the response of brain tumors to alkylating agents. We showed that the methylation of MGMT promoter was correlated with a poor outcome in gliomas treated with BCNU-based chemotherapy [9]. However, it is unclear the effect produced by alkylating agents according to MGMT expression. Rather, drug resistance likely involves the altered expression of a diverse group of genetic factors influencing various biochemical pathways.

An increase in the understanding of molecular biological properties of anticancer agents will lead to the development of mechanism-based therapeutic strategies. In this context, changes in the RNA expression level, which are closely related to the amount of protein product, and thus the biochemical activity, may be considered as the first evidence for a gene with resistance potential. Meanwhile MGMT has been demonstrated to exert a role in the resistance to alkylating agents, other possible mechanisms can contribute to decrease the response to chemotherapy and explain the molecular

events underlying the effect of BCNU. In the same way, several biochemical pathways that include MGMT protein must be the responsible of resistance to BCNU in gliomas. DNA microarray analysis allows the simultaneous and rapid analysis of the expression of thousands of genes [10–13], and, in turn, provides an opportunity for determining the effects of anticancer agents on different cell lines. To better understand the precise molecular mechanisms by which BCNU exerts its effect on glioma cell lines, we utilized a DNA microarray. We will look for mRNA levels of 3800 genes and determine the gene expression profile of MGMT-deficient glioma cell lines and glioma cell lines expressing this protein treated with BCNU.

Materials and methods

Cell lines and cell culture

T98G, a human glioma cell line expressing MGMT protein, was purchased from the American Type Culture Collection (Rockville, MD). T98G was cultured in MEM media with L-Glutamine (ATCC) supplemented with 10% bovine serum (Life Technologies, Inc.), 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37 °C. A172 (ATCC), MGMT-deficient glioma cell line, was cultured in DMEM media with L-Glutamine (ATCC) supplemented with 10% bovine serum, 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37 °C.

IC50 determination: neutral red assay

BCNU was purchased from Brystol-Myers Pharmaceuticals. A stock solution 50 mM was prepared by dissolving BCNU in DMSO and stored at −70 °C to ensure drug stability. Subsequently, BCNU dilutions (1 mM to 20 μM) were prepared and added to culture plates for IC50 determination.

Cells were resuspended in culture medium and 100 μl of cell suspension (10000 cells) were cultured for 18 h to assure similar attachment of all cells. BCNU dilutions or DMSO (vehicle control) were added in 100 μl of culture medium and incubated for 72 h. After washing with PBS, the cells were incubated at 37 °C with neutral red during 1.5 h. The dye was then extracted from the cells by addition of 100 μl 0.05 M NaH_2PO_4 in 50% ethanol. Optical density was read at 540 and 650 nm as a reference wavelength using a microtiter plate reader (Organon Teknika). The spectrophotometer was blanked on the first column of control wells containing solvent solution alone.

The IC50 was determined in triplicate by curvilinear regression analysis.

DNA microarray analysis for gene expression profile

T98G (MGMT+) and A172 (MGMT−) were treated at their respective IC50 concentrations of BCNU (460 and 130 μM, respectively) when similar culture confluence was achieved (70%). As treatment control, vehicle (DMSO) was used. After 24 or 48 h culture with BCNU or vehicle alone, cells were washed with phosphate buffered saline (pH 7.4), tripsinized and scraped from plate.

Total RNA from each sample was isolated by using RNeasy Midi Kit and RNase-free DNase Set (QIAGEN, Valencia, CA) according to the manufacturer's protocols. The concentration was calculated spectophotometrically and RNA was adjusted to 1μg/μl. Quality control of RNA were checked by electrophoresis and ethidium bromide staining on a 2% agarose gel.

Preparation of the cDNA probes was performed using the Micromax system (NEN, Perkin Elmer, Boston, MA) according to the manufacturer's protocol. Briefly, 2 μg of RNA from control cells was labeled with Biotin-11-dCTP and 2 μg of RNA from BCNU-treated cells was labeled with Fluorescein-12-dCTP. Mix probes resuspended in 50 μl of GlassHyb Hybridization Solution (Clontech, Palo Alto, CA) were pre-heated to 50 °C and applied to Atlas Glass Human 3.8 Microarray (Clontech) where 3800 known different genes are included.

After hybridization at 50 °C for 16 h in a slide cassette (Telechem, Sunnyvale, CA), slides were washed sequentially in a series of solutions with increasing stringency: 0.5 × SSC, 0.01% SDS (5 min, room temperature); 0.06 × SSC, 0.01% SDS (5 min, room temperature); and 0.06 × SSC (2 min, room temperature). Immediately after washing, the presence of biotin and fluorescein labeled cDNAs on the microarray was detected using a fluorescent streptavidin or fluorescein-antibody conjugates and TSA detection according to the manufacturer's protocol with appropriate modifications (Micromax). Post-hybridization detection techniques offer some major advantages over direct labeling procedures. Direct labeling generally reduces the efficiency of enzymatic synthesis and can introduce fluorescence-dependent labeling bias, mandating the use of larger amounts of RNA and 'dye-swapping' strategies to obtain strong signals and avoid experimental artifacts. Unincorporated dyes can also increase fluorescent background if those are not removed before hybridization. Post-hybridization detection methods can reduce or eliminate these complications and provide more reproducible results.

Microarray data normalization and analysis

The GMS 418 scanner, a confocal scanning instrument containing 2 lasers that excite cyanine dyes at 635 nm for Cy5 and 532 nm for Cy3, and a high-resolution photo multiplier tubes (10 μm pixel size), was used for scanning the hybridized microarrays. After image acquisition, the scanned images were imported into 'ImaGene 4.1' software (BioDiscovery) to quantify the signal intensities. Data sets of spots not recognized by the ImaGene analysis software were excluded from further considerations (empty, poor and negative spots). We also removed data from spots identified as visible flawed (<15% in all arrays). The fluorescent median signal intensity for each spot was calculated using local median background substraction. We then normalized

the ratio data using a robust local-linear regression 'lowess' and filtered log ratios <−2 or >2. A gene was considered to be differentially expressed when the difference in fluorescent intensity between the two fluorochromes is greater than fourfold. Genes that were differentially expressed were further divided into smaller groups based on their functions reported in the literature, such as promotion or inhibition of cell signal transduction, transporter, structural proteins, proliferation, DNA repair, cell cycle progression and apoptosis.

Real-time RT-PCR

The total RNA prepared for microarray was also used for real-time RT-PCR. 2 μg of total-RNA from each sample was used to generate cDNA using the Taqman Reverse Transcription Reagent kit (PE Applied Biosystems, Foster City, CA) according to manufacturer's protocol.

Each cDNA sample was analyzed in triplicate using the ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). Quantitative assessment of DNA amplification was detected using the SYBR Green PCR Master Mix (Applied Biosystems). The Q-RT-PCR reactions were carried out in a total volume of 25 μl using a primer concentration of 300 nM. Primers for five candidate genes (*SNAPC1*, *SNAPC3*, *SNRPB2*, *SMARCD1* and *GTF2F1*) were designed to cross intron–exon boundaries to distinguish PCR products generated from genomic vs. cDNA template (Table 1). All primers were designed using the software Primer Express 1.0 (Applied Biosystems). For thermal cycling, the following conditions were applied: 10 min at 95 °C, then 45 cycles of 15 s at 95 °C and 1 min a 59 °C, followed by a final heating step up to 95 °C within 20 min (heating rate 1.75 K/min) to obtain melting curves of the final Q-RT-PCR products using the ABI Prism Dissociation Curve Software (Applied Biosystems). This is necessary because SYBR Green fluorescence may also be derived from side products such as primers dimmers. Each product was confirmed to be a single peak and product size was checked on an agarose gel. To normalize the amount of sample cDNA, 2 endogenous control transcripts of 'housekeeping genes' coding for glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) and 18s-*rRNA* were used.

Fold induction was calculated using the formula $2^{-\Delta\Delta Ct}$, where ΔCt = target gene −Ct-'housekeeping' gene Ct, and $\Delta\Delta Ct$ is based on the mean ΔCt of respective control (non-BCNU treated). The Ct value is determined as the cycle at where the fluorescence signal emitted is significantly above background levels and is inversely proportional to the initial template copy number.

Real-time PCR using 5′ nuclease 'Taqman' for MGMT detection was carried out as described above for SybrGreen with the following exceptions: Taqman Universal PCR Master Mix (Applied Biosystems), containing ROX to normalize emissions, and FAM-labeled *MGMT* probe (see Table 1) were used. Primers and probe used for amplification and detection *GAPDH* gene were purchased by Applied Biosystems (TaqMan GAPDH Control Reagents Kit). *MGMT* expression were normalized to the Ct of *GAPDH* using ΔCt (difference in threshold cycles for target and reference genes).

Results

Drug sensitivity

To explore the potential functional consequences of differential gene expression of BCNU treatment in glioma cell lines, we first examined the effect of BCNU treatment on the viability of A172 and T98G cell lines. Cells were treated with increasing concentrations of BCNU and cell viability was determined using the

Table 1. Primers used for real time RT-PCR

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Probe (5′–3′)-TAMRA
Taqman specific detection			
GAPDH[a]	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	JOE-CAAGCTTCCCGTTCT-CAGCC
MGMT	GCTATCGAAGAGTTCCCCGTG	TCGTTCACCAGACAGGTGTTATG	FAM-CGGCTCTTCACCAT-CCCGTTTTCC
Sybergreen detection			
GAPDH	TCGGAGTCAACGGATTTGG	GGCAACAATATCCACTTTACC-AGAGT	–
RNAr18s	GCCGCTAGAGGTGAAATTCCTT	GAAAACATTCTTGGCAAATGCTTT	–
SNAPC1	CTTCCAGGAGACGGACAGTGTAC	AAATTTCTCATTCTGCCACAG-AAGATA	–
SNAPC3	CCTCCAGATTGGTGGTGAATTC	GCTGATTTGTATAGGTCTTT-GCTGAT	–
SMARCD1	GCCTTGAAACGTCCCATCA	GCATCTGACTTAGCCGGATTG	–
SNRPB2	CTGCAACAACCACAAACAAAAAG	TGGTGTTGAATTTCCTTGGGTAT	–
GTF2F1	TTTTAATGCAGCCGACAAAGTC	TTGGTAGATTTTCTTGTTG-CTCAAGT	–

[a] Primers and probe used for amplification and detection GAPDH gene are from the TaqMan GAPDH Control Reagents Kit (Applied Biosystems).

neutral red assay. Human glioma cell lines deficient for MGMT expression were more sensitive to BCNU than T98G cell line expressing MGMT. The IC50 for BCNU in human glioma cell lines expressing MGMT protein is 3.5-fold-higher than human glioma cell lines deficient for this protein (460 μM vs. 130 μM). This result is in concordance with the previous results of Weller et al. [14]. According to these studies, A172 glioma cell line is more chemosensitive than T98G.

In order to elucidate if MGMT expression could be induce by BCNU treatment, we analyzed the expression of this protein in both cell lines treated with BCNU for 24 and 48 h and control by real-time RT-PCR. As shown Figure 1, we did not detected mRNA expression of *MGMT* in A172, neither in the control nor in treated cells. In this sense, it has been reported by Gomi et al. [15] that A172 resistant to another nitrosourea (ACNU) does not express MGMT. Moreover, the expression of DNA polymerase β, another protein involved in DNA repair, is increased in ACNU-resistant cells (A172R). In contrast, looking at the T98G glioma cell line, the control line express this protein but treatment with BCNU do not induce a greater expression of *MGMT*. These results suggest that other pathways could be implicated in BCNU resistance.

Regulation of mRNA expression by BCNU treatment

To identify genes whose expression was changed by BCNU treatment, we analyzed expression profiles of A172 and T98G cells in response to BCNU in a time-course experiment. We used an oligonucleotide microarray of 3800 genes.

Initially, we analyzed time-dependent changes in the expression profile of A172 glioma cell line (deficient for MGMT) in response to BCNU. After data filtering, we found a total of 23 genes showing a greater than 4-fold change after 24 h of BCNU treatment. Among these, 14 genes were up-regulated and 9 were down-regulated. A longer drug's exposure time increased the number of changing genes. After 48 h, a total of 49 genes show a greater than 4-fold change in expression with down-regulation of 32 genes and up-regulation of 17 genes (Figure 2). Seven genes up-regulated and 2 genes down-regulated at 24 h remained altered after 48 h and 40 new genes changed after a longer exposure time. According to their classification by biological function the most important pathways altered are the mechanisms of transcription (Table 2).

Treatment with BCNU in T98G glioma cell lines (expressing repair-DNA protein MGMT) induces greater gene expression changes, mainly after 48 h (Figure 2). Figure 3, shows the common altered genes in both cell lines. In cell lines expressing MGMT 24 h after the treatment a greater number of genes were down-regulated than in A172 cell line. A total of 43 genes showed a greater 4-fold change in expression profile after 24 h of BCNU treatment. Out of these 43, 9 genes were up-regulated and 34 were down-regulated. After 48 h treatment, the number of genes up-regulated were 19 and those down-regulated increased to 159 genes. When we classify the genes exhibting changes after BCNU treatment according to their known function, we found that BCNU affected the expression of several genes that are involved in the transcription process (Table 3). Among these genes, *SNAPC1*, *SNAPC3* and *SNRPB* regulate directly transcription mechanism through nucleation of both RNA polymerase II and RNA polymerase III transcription initiation complexes [16].

Target verification by Q-RT-PCR

To verify the results of the microarray analysis, five genes were chosen to determine their expression profiles by Q-RT-PCR. The same RNA samples used in microarray hybridizations were tested by quantitative RT-PCR. The specificity of the amplified products were tested during each experiment by plotting a melting slope from progressive final denaturation. The results of RT-PCR analysis for these selected genes were in agreement with the microarray data. These results,

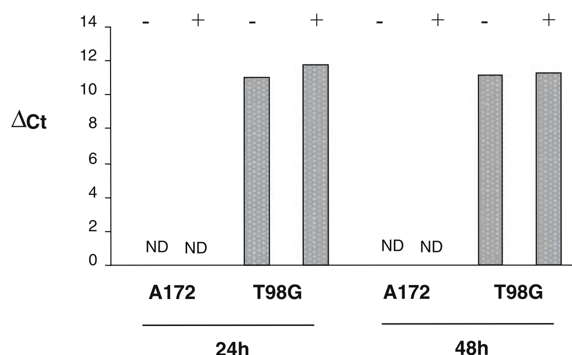

Figure 1. mRNA expression of MGMT gene in A172 and T98G glioma cell lines, before (−) and after (+) BCNU treatment. As expected, we did not detected MGMT in A172 glioma cell line. In T98G we detected MGMT expression but BCNU treatment do not increase its expression.

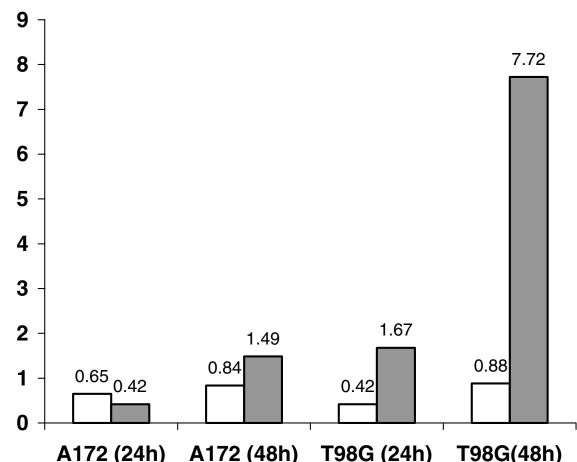

Figure 2. Percentage of total evaluated (3800 genes) that are been found up (open bars) or down-regulated (solid bars) after 24 or 48 h treatment with BCNU in A172 (MGMT-deficient) and T98G (MGMT +) glioma cell lines.

Table 2. Gene expression changes in A172 glioma cells (MGMT-deficient) treated with BCNU during 24 and 48 h

Biological process	24 h		48 h	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Metabolism	ARSC1 **SMPD2**	FNTA HADHSC SLC21A3	SIAT1 SIAT8 **SMPD2**	ATP5D EPHX1 YWHAH
Cell cycle				FYN PNUTL1
Signaling transduction	AVPR1A **GNRHR** GDI1	**INPPL1** RCN1	**GNRHR** GDI2	ADRA1B ADCYAP1 **INPPL1** IRS1 PPM1G MAP4K2
Structural protein	**ECM2**	**ACTN1**	**ECM2**	ACTC **ACTN1** FBN1 KRT17 PFN1 TUBG1 WASPIP
Communications proteins				NEGF2 AMH INSL3 NRTN
Transcription	**SNAPC1** **SNRPB2** **SNAPC3** **SMARCD1**	**GTF2F1** TLE3	ESRRA RNASE4 **SNAPC1** **SNRPB2** **SNAPC3** **SMARCD1** GCMA	**GTF2F1** HMGN2 SHOX
Transduction	EEF1G EIF2B1	GOLGA2		LOXL1
DNA repair				PMS2L11
Apoptosis			BCL10	
Transporters			SLC10A1	AQP8 ATP1A3
Receptors	**CD80**		**CD80** LAIR1	ITGA3
Functionally unclassified	PSCD1		SNCG	LRN INSIG1

The fold-change was in all genes < -2 or > 2. Genes with changed expression at 24 and 48 h in bold.

therefore, support the findings obtained from microarray experiments and suggest that BCNU treatment mainly down-regulated an important number of genes.

Discussion

There has been growing interest in the use of microarray, which makes possible to analyze the expression of a large number of genes simultaneously. The gene expression profiles of various types of cancers have been analyzed using microarray [17–19]. The alterations of gene expression profiles by several anticancer agents have been also reported [20–22].

Carmustine (BCNU) is an alkylating chemotherapeutic agent used primary for the treatment of human brain tumors. BCNU induce DNA crosslinking arises from its alkylation reaction and contributes to cell killing and antitumour activity of this drug [23,24]. The MGMT protein rapidly reverses alkylation at the O^6 position of guanine, therefore MGMT causes resistance to alkylating agents as we described in a previous article [25,26]. Previous studies have shown that BCNU-resistance in gliomas is associated with MGMT expression. However, little is known about the gene expression profile in glioma cancer cells after BCNU treatment.

In this study, we utilized the microarray technology, studied 3800 known genes, to determine the changes in

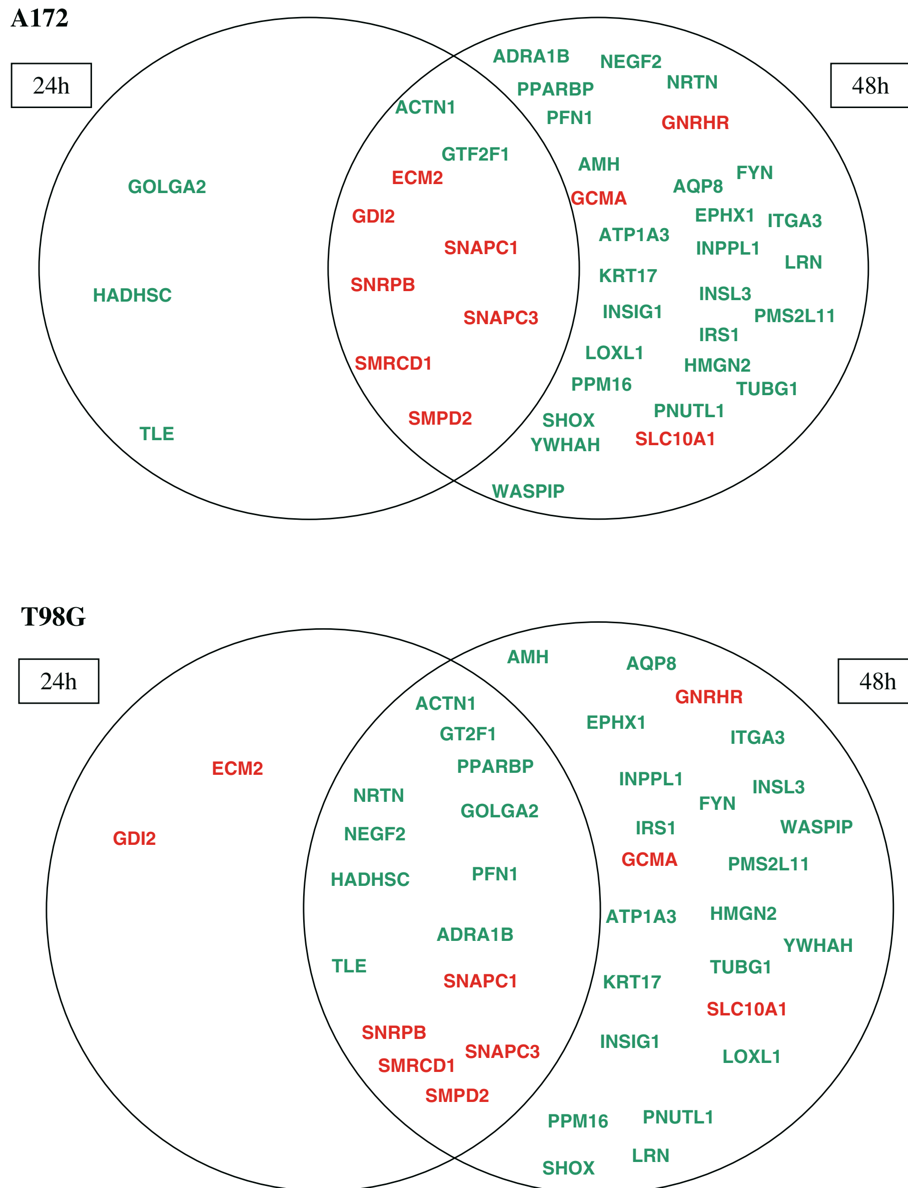

Figure 3. Diagram representation of time-related changes in expression profile in A172 and T98G cell lines. Each circle represents a exposure time of treatment with BCNU (24 and 48 h). Genes represented in red correspond to up-regulated expression and in blue to down-regulated expression. Genes are identified by their UniGene symbol (http://www.ncbi.nlm.nih.gov/Unigene).

gene expression profiles of glioma cell lines according to *MGMT* expression exposed to BCNU. Our results from oligonucleotide microarray have been used to analyze the cellular response to BCNU treatment. Cellular response to any antiproliferative agents involve modulations of complex pathways that ultimately determine whether a cell survives or dies. Molecular response of glioma cell lines to BCNU are complex and are likely to be mediated by a variety of regulatory pathways. Mainly, these genes have specific functions in signal transduction, transcriptional regulation, cell proliferation and cellular metabolism.

The most frequent site of alkylation in DNA is the O^6 position of guanine. BCNU generates highly reactive intermediates that transfer chloroethyl groups to a nucleophilic site on one DNA strand, then the resulting chloroethyl monoadducts react with a second nucleophilic site via displacement of chloride to form an ethylene bridge between the two nucleophilic sites. The cross-linking of double-stranded DNA by alkylating agents is inhibited by the cellular DNA-repair protein O^6-methylguanine-DNA methyltransferase (MGMT). MGMT brings about the repair of O^6-alkylguanine in double-stranded DNA by transferring the alkyl group from the DNA to an internal Cys residue within the conserved active site PCHRV in the MGMT protein (Figure 4). Only one alkyl group per protein molecule can be transferred though a stoichiometric reaction. In these same way, it is now fully established that the DNA adduct, O^4-methylthymine (m^4T), which is a very minor product the reaction of DNA with methylating agents, can also be repaired by MGMT. This adducts promote alternative mechanisms of transcriptional machinery activating the small nuclear RNA-activating complex (*SNAPC*). In human cells, *SNAPC* binds to the proximal sequence element in both RNA polymerase II- and

Table 3. Gene expression changes in T98G glioma cells (MGMT+) treated with BCNU during 24 and 48 h

Biological process	24 h		48 h		
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	
Metabolism	FNTA	**HADHSC**	**SMPD2**	ALAD	COX6A2
	SMPD2	**HAGH**	UGT8	ALOX15B	FTH1
		PZP		ASMTL	IDS
				ATP6V0C	MAT1A
				B4GALT5	MGAT3
				CYP19A1	MVD
				FTL	PFKM
				EPHX1	PCBD
				GAMT	NDUFC1
				CRAT	PCYT1A
				CA6	PFKFB2
				HADHSC	SHMT2
				CKM	YWHAH
				HAGH	CST5
Cell cycle		TRAF1		BECN1	
				PNUTL1	
				CDK8	
				MAD1L1	
				FYN	
Signaling transduction	GDI1	**ADRA1B**	GNRHR	**ADRA1B**	GRB7
		GPR37	PIK3R3	ADCYAP1	CRABP1
		RGR		INPPL1	GPR32
		RIT2		IRS1	GHSR
				PPM1G	MYD88
				MAP4K2	MAPK10
				AGTRL1	PTPN3
				AKT2	**RGR**
				APCL	STAM
				BAIAP2	Zyxin
				BAIAP3	CALB2
				BLK	GPR25
				CHRM4	DVL1
				GPR8	
Structural protein	ECM2	**ACTN1**	TUBB	**ACTN1**	**ARGBP2**
		ARPC2		FBN1	**COL9A1**
		ARGBP2		KRT17	GFAP
		COL9A1		**PFN1**	ACTG2
		PFN1		TUBG1	KRTHA1
				WASPIP	MYBPH
				ARPC2	SGCA
				CRYBB1	LAMB3
				H2AX	PRM2
Communication proteins		**ARTN**	SCYA2	ADAM15	INSL3
		GAS		AMH	LAD1
		NEGF2		ADAMTS4	LRMP
		TNFRSF11B		**ARTN**	PAEP
		NRTN		BMP7	PRL
				CTF1	**NRTN**
				GRN	TAC1
				NEGF2	VGF
Transcription	**SNAPC1**	**GTF2F1**	**SNAPC1**	**GTF2F1**	TAF3B2
	SNRPB2	**TLE3**	**SNRPB2**	HMGN2	CEBPB
	SNAPC3	PPARBP	**SNAPC3**	SHOX	HCFC1
	SMARCD1	**IKBKG**	**SMARCD1**	BICD1	EZH2
		DDX16	GCMA	FOXJ1	HOXA4
				CDX4	LMX1B
				IER3	MYF3
				MYOG	POLR2E
				NPAS1	TCF1
				PBX2	RENT1
				NKX2B	SRY
				TCF21	**TLE3**

Table 3. Continued

Biological process	24 h		48 h		
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	
				SRP14	ZNF151
				SFPQ	PAX8
				IKBKG	
Transduction		STXBP2	GOLGA4	LOXL1	STX1B
		GOLGA2	CLTCL1	**GOLGA2**	ITGB4BP
				FKBP1A	KIF5A
				PCOLCE	
DNA repair		RAD51		PMS2L11	
				RAD9	
				RAD23A	
Apoptosis				TRAF1	
				DDX16	
Transporters		**MSR1**	SLC10A1	AQP8	KCNH2
		SLC30A3	SLC6A2	ATP1A3	ITIH1
			SLC16A2	ATP4B	MUC2
				MSR1	**SLC30A3**
Receptors		ICAM5	CNTNAP1	ITGA3	**GABRA2**
			GPR65	GP1BB	CD79A
		GABRA2	CTNNAL1	GABRA6	CHRNG
				CHRNB3	CEACAM3
				CD68	PCDHGC3
				CD151	OMP
Functionally unclassified	TEGT	**APEG1**		A2LP	INSIG1
		TM4SF7		**APEG1**	ISLR
		SPRR2B		DNASE1L2	TEGT
				LMX1B	**SPRR2B**

The fold-change was in all genes <-2 or >2. Genes with changed expression at 24 and 48 h in bold.

III-dependent small nuclear RNA gene promoters [27]. An increase of these transcription factors essential for RNA polymerase II and III activation is not enough for maintaining cellular transcription mechanisms after treatment with alkylating agents. Moreover, after 48 h of BCNU treatment down-regulation of a large number of genes is evident. Our results suggest that BCNU treatment in glioma cell lines seems to induce the expression of human SW1/SNF-like chromatin-remodeling protein complex (*SMARCD1*), also called BRG1-associated factors [28]. Alkylating agents could promote changes in the chromatin remodeling and transcriptional activation. The SWI/SNF protein complex interacts with chromatin and thereby facilitates the function of transcriptional activators. Our data show that the treatment of BCNU would activate the nucleosome disruption. This effect is evident a 24 h and more intensive after 48 h of BCNU exposure. The results obtained in T98G with a IC50

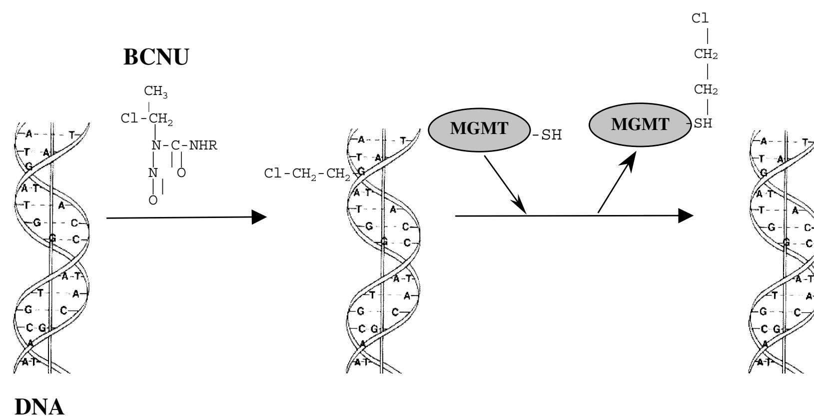

Figure 4. Scheme of the mechanism of DNA interstrand by BCNU and mechanism of action of MGMT. BCNU generates highly reactive intermediates that transfer chloroethyl groups to a nucleophilic site (O^6-guanine) on one DNA strand. The MGMT protein irreversibly transfers the alkyl group of the O^6 position of guanine to a cysteine residue within the conserved active site PCHRV of the MGMT protein. There is no cleavage of the DNA and the guanine is restored to its original unaltered state.

clearly greater than in A172 are very similar. Alternative transcription mechanisms could be activated to avoid the DNA injury produced by BCNU as Gomi et al. [15] proposed in ACNU resistant cells (A172R).

MGMT action is not enough for reversing damage induced by BCNU at the concentration tested and other repair mechanisms seem useless because the damage induced by alkylating agents is mainly base-specific. BCNU treatment down-regulated also genes involved in DNA-repair such as *PMS2L11*, *RAD9* or *RAD23A*.

In summary, we have analyzed the gene expression profiles of A172 and T98G glioma cell lines exposed to different BCNU concentrations according to their MGMT profile. This drug altered the expression of many genes that are related to transcription regulation, cell signaling transduction and other essential cellular pathways, suggesting pleiotropic effects of this compound. BCNU-induced regulation of these genes may be exploited for devising mechanism-based chemopreventive or therapeutic strategies for brain tumors. Up-regulated transcription factors and chromatin regulators could activate alternative pathways to overcome the DNA injury produced by BCNU avoiding the entry to apoptosis. However, further in-depth studies are required to investigate the effects of BCNU on the regulation of important cellular molecules at the protein level to examine the effects of BCNU with cellular functions.

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