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ACCELERATED COMMUNICATION

Mechanisms of Resistance to (2-Chloroethyl)-3-Sarcosinamide-1-Nitrosourea (SarCNU) in Sensitive and Resistant Human Glioma Cells

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SUMMARY

Resistance to (2-chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU), an experimental antitumor compound, was investigated in the sensitive SK-MG-1 cells and the 20-fold more resistant SKI-1 human glioma cells [which are 3-fold more resistant to 1,3,bis(2-chloroethyl)-1-nitrosourea (BCNU)]. The transport of SarCNU was examined by utilizing tritiated sarcosinamide. Sarcosinamide uptake into SK-MG-1 cells is via the catecholamine carrier that accommodates epinephrine. Dixon plot analysis of SarCNU inhibition of sarcosinamide uptake reveals that SarCNU is also accommodated by this carrier. The uptake of 0.5 mM [^{3}H]sarcosinamide was temperature dependent, with similar levels of intracellular sarcosinamide accumulating at steady state in both cell lines. The uptake of sarcosinamide in SKI-1 cells obeyed Michaelis-Menten kinetics over a 200-fold range of concentrations with a K_m of 1.52 ± 0.151 mM and V_{max} of 0.659 ± 0.066 nmol/10^6 cells/min. This represents a more than 5-fold decrease in the uptake affinity and a more than 4-fold increase in the transport capacity compared with SK-MG-1 cells (K_m = 0.282 ± 0.041 mM; V_{max} = 0.154 ± 0.024 nmol/10^6 cells/min). The initial rate of sarcosinamide uptake is similar in both cell lines. Dixon plot analysis confirmed that SarCNU is a competitive inhibitor of sarcosinamide transport in SKI-1 cells with a K_i of 17.5 mM, which is more than 5-fold greater than the K_i obtained in SK-MG-1 cells. The steady state accumulation of SarCNU is significantly reduced by 47% in SKI-1 cells compared with the SK-MG-1 cells (cell to medium ratios of 0.65 ± 0.11 and 1.22 ± 0.08, respectively) ($p < 0.005$). The accumulation of BCNU was comparable in the two cell lines. Since the V_{max} of sarcosinamide (SarCNU) uptake is increased in the SKI-1 cells, the decrease in intracellular SarCNU is not related to decreased drug influx via the catecholamine carrier in SKI-1 cells. The efflux of tritiated sarcosinamide was temperature dependent and similar in both cell lines, with 54 and 58% of sarcosinamide being freely exchangeable in SKI-1 and SK-MG-1 cells, respectively. SarCNU efflux may or may not be altered. Since the expression of mdr is higher in the sensitive cells, it is unlikely that increased efflux of SarCNU mediated by the P-glycoprotein is responsible for drug resistance. A significantly lower percentage of DNA cross-links was observed in the resistant glioma cells 24 hr after SarCNU treatment, with the total area under the curve for DNA crosslinks reduced to 59% of that measured for the sensitive cells. Both cell lines exhibited low levels of [^{6}O]alkylguanine DNA alkyltransferase, which is characteristic of the Mer$^-$ phenotype. The total activity of the base excision enzyme, 3-methyladenine DNA glycosylase, was comparable in the two cell lines. High-performance liquid chromatography (HPLC) analysis revealed that the removal of 7-methylguanine and 3-methyladenine by the glycosylase was similar in both cell lines. The expression of the excision repair gene, ERCC-1, was not increased in the resistant cells. The expression of the class mu glutathione transferase (GST) was 20% higher in the resistant cells. However, GSH levels were 8-fold higher in the sensitive cells.

These results indicate that resistance to SarCNU in SKI-1 cells correlates with reduction in intracellular drug accumulation and DNA cross-links without alterations in drug influx via the catecholamine carrier or DNA repair enzymes. Detoxification by GST mu may play a minor role in resistance to SarCNU. Additional mechanisms such as alternative DNA repair, decreased drug influx via a carrier other than the catecholamine carrier, and/or increased drug efflux may be responsible for the decreased SarCNU accumulation and decreased DNA cross-linking.

SarCNU is a novel CENU linked to the amide of methylglycine (sarcosinamide) (1). SarCNU was previously shown to be more active than BCNU in primary and in cultured human glioma cells *in vitro* and in human glioma cells transplanted in nude mice (2, 3). Relative to other CENUs which passively diffuse into cells, the transport of SarCNU was found to be altered by virtue of the sarcosinamide moiety (4). Sarcosinamide uptake was characterized in human glioma SK-MG-1 cells and was determined to proceed via a sodium- and energy-independent catecholamine carrier. The characteristics (K_m/V_{max}) of sarcosinamide uptake were similar to those of epinephrine. Dixon plot analysis revealed that SarCNU competitively inhibited sarcosinamide uptake, with a K_I of 3 mM compared with a K_m of 280 μM for sarcosinamide. Moreover, the V_{max} of

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ABBREVIATIONS: SarCNU, (2-chloroethyl)-3-sarcosinamide-1-nitrosourea; BCNU, 1,3,bis(2-chloroethyl)-1-nitrosourea; CENU, chloroethylnitrosourea; Gatase, [^{6}O]alkylguanine DNA alkyltransferase, GST, glutathione transferase; AUC, area under the curve; CFA, colony-formation assay; GSH, glutathione; MNU, methylnitrosourea; PAG, phosphate buffered saline with 0.7% BSA, 0.25% dextrose, and 0.001% phenol red; HPLC, high-performance liquid chromatography; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DTT, dithiothreitol.

SarCNU inhibition of sarcosinamide uptake derived from the Dixon plot is similar to the V_{max} of sarcosinamide uptake, indicating that SarCNU inhibition of sarcosinamide is purely competitive (5). The evaluation of the *in vitro* cytotoxicity of SarCNU in cultured human glioma cells led to the identification of one cell line (SKI-1 cells) which is highly resistant to SarCNU (2). To date, resistance to CENUs has been associated with increased drug inactivation by glutathione transferase (6) and/or increased repair of a specific DNA adduct by [^{6}O] methylguanine-DNA methyl transferase (Gatase) which prevents the formation of a cytotoxic DNA interstrand cross-link (7–9). Alternative mechanisms for repair of DNA lesions have been described. In one instance, DNA adducts at the ^{6}O position of guanine were shown to be repaired by nucleotide excision in Gatase-deficient cells (10). Additionally, a preliminary report indicates that resistance to the alkylating agent cisplatin may be related to the expression of the excision repair gene ERCC-1 (11). Finally, recent studies suggest that DNA glycosylases may also mediate the repair of CENU-induced DNA lesions (12, 13). The existence of a specific transport mechanism for SarCNU raises the possibility that resistance to this CENU may be related to the uptake capacity in glioma cells.

The objective of this study was to compare the transport of tritiated sarcosinamide in resistant and sensitive human glioma cells in order to determine whether changes in this parameter may contribute to resistance. Direct studies with SarCNU were not possible because this compound is not available as a radiolabeled drug. We previously showed that the transport of SarCNU is specific for sarcosinamide; therefore, the latter compound is an appropriate substrate for this study (4, 5). The formation of DNA cross-links, activities of Gatase and glycosylase, and the expression of RNA for GST mu, ERCC-1, and mdr1 were compared in these cells to evaluate their potential roles in resistance to SarCNU.

Experimental Procedures

Drugs. SarCNU (NSC# 364432) was generously provided by Dr. T. Suami, Keio University, Japan. The drug was dissolved in 0.001 M citrate buffer, pH 4.0, and stored at −20°.

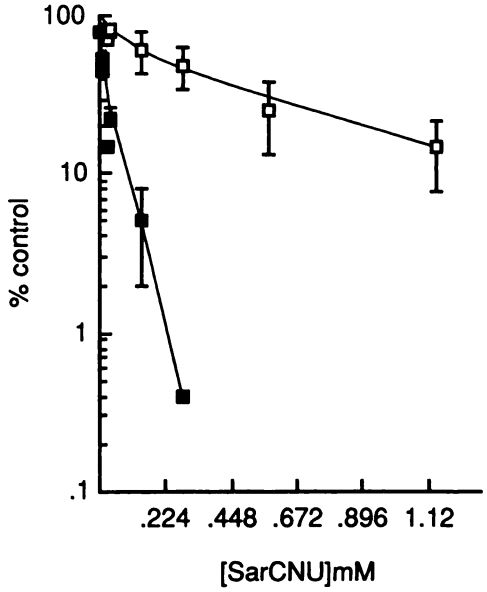

Fig. 1. Survival of SK-MG-1 cells (■) and SKI-1 cells (□) after treatment with SarCNU in PAG medium for 1 hr at 37°. Cytotoxicity was measured as the percentage of colony growth of untreated controls in the CFA. The data on the graph represent the means ± standard errors for three or more separate experiments.

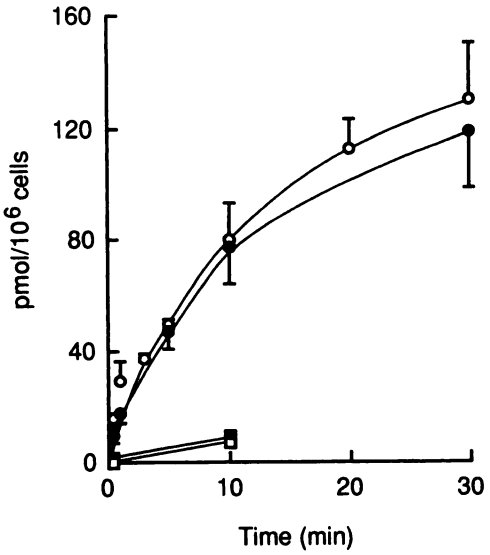

Fig. 2. Time course of the uptake of 0.05 mM [^{3}H]-*N*-sarcosinamide in SK-MG-1 (○) and SKI-1 (●) cells at 22° and in SK-MG-1 (□) and SKI-1 cells (■) at 0°. The uptake is expressed as the picomoles of tritiated sarcosinamide per 10^6 cells. The data on the graph represent the means ± standard errors for five separate experiments.

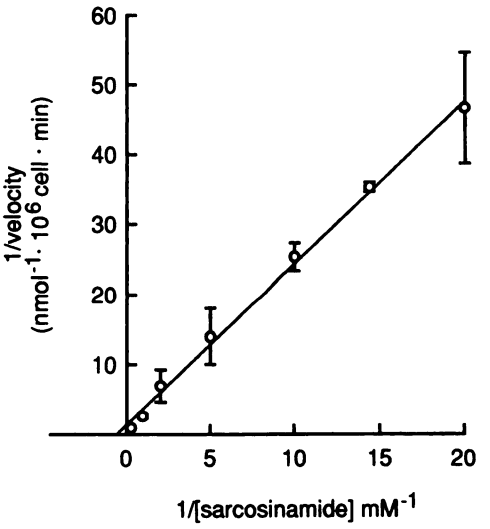

Fig. 3. Lineweaver-Burk plot of the initial uptake velocity of 0.05 to 4 mM tritiated sarcosinamide at 22°. The data represent the means ± standard errors for four separate experiments. The results were analyzed by linear regression and the equation is $y = 2.31x + 1.52$, with a correlation coefficient of 0.998.

TABLE 1

Kinetic parameters in human glioma cells

The kinetic constants K_m and V_{max} were determined from Lineweaver-Burk plots for tritiated sarcosinamide as described in Materials and Methods and in a previous study (5).

Cell line	V_{max}*	K_m*
	nmol/10^6 cells/min	*mM*
SK-MG-1	0.154 ± 0.024	0.282 ± 0.041
SKI-1	0.659 ± 0.066	1.52 ± 0.151

* Mean ± standard error for at least four separate experiments.

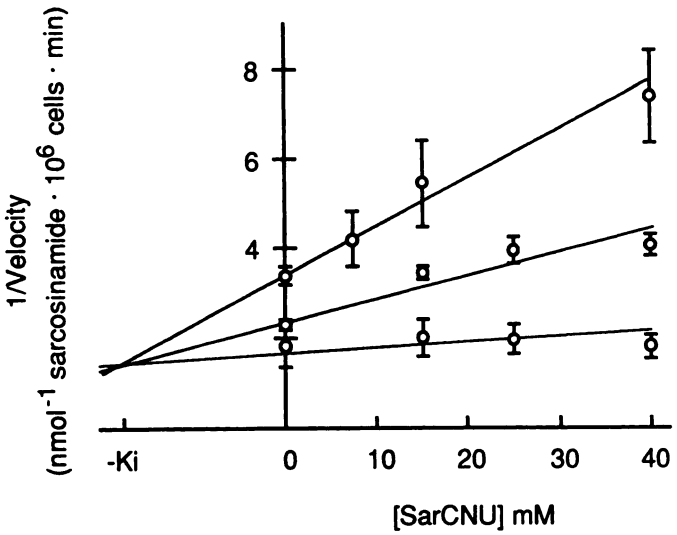

Fig. 4. Dixon analysis of SarCNU inhibition of sarcosinamide transport in SKI-1 cells. The 30-sec uptakes of 0.50, 0.75, and 1.0 mM tritiated sarcosinamide in media containing 7.5 to 40 mM SarCNU were measured in SKI-1 cells at 22° as described in Materials and Methods. The results represent the means ± standard errors for three separate experiments. The plots from top to bottom represent 0.50 to 1.0 mM sarcosinamide.

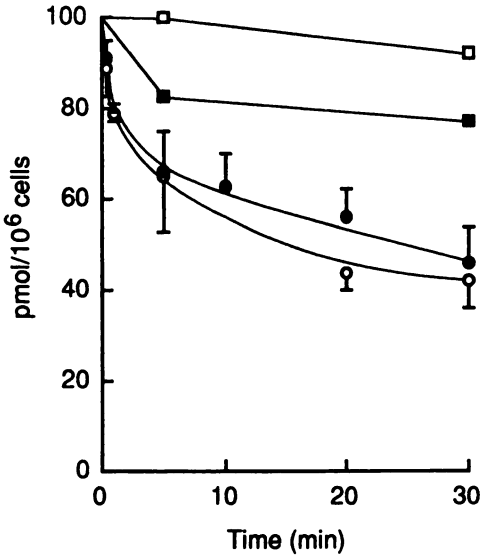

Fig. 5. Time course of efflux of tritiated sarcosinamide in SK-MG-1 (●) and SKI-1 (○) cells at 22° and in SK-MG-1 (■) and SKI-1 cells (□) at 0°. Efflux is expressed as picomoles of tritiated sarcosinamide per 10^6 cells. The data represent the means ± standard error for three separate experiments.

Materials. Tritiated [^{3}H]-*N*-sarcosinamide was custom synthesized by Amersham Laboratories, Buckingham, England, to a specific activity of 1.05 Ci/mmol. Sarcosinamide HCl, sulfanilamide, [1-naphtyl]-ethyldiamine dihydrochloride (Britton-Marshall reagent), glycyl glycine HCl, and sulfosalicylic acid were purchased from Sigma Chemical Co. Tritiated water (90 μCi/mmol) and [*carboxyl*-^{14}C]inulin (3.2 mCi/mmol) were purchased from Amersham Corporation. McCoy's 5A modified medium, fetal calf serum (FCS) and Dulbecco PBS were obtained from Grand Island Biological Co., Grand Island, NY; bovine serum albumin (BSA) (Fraction V, powder; low-salt and salt-free fractions) was from Miles Laboratories; Versilube F-50 silicone oil was from Nessa Products.

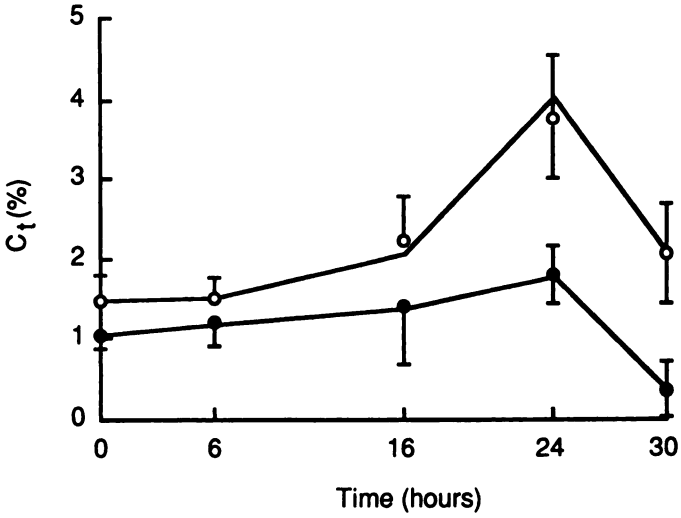

Fig. 6. Time course of the percentage of SarCNU-induced cross-links (C_t) in SK-MG-1 (○) and SKI-1 (●) cells following treatment with 0.28 mM SarCNU for 1 hr at 37° as described in Materials and Methods. The data represent the means ± standard errors for six separate experiments. The curves generated by the points from 0 to 24 hr fit best with a log curve for SK-MG-1 cells ($y = 0.00004175x^{3.47}$; $r = 0.99$) and an exponential curve for SKI-1 cells ($y = 1.05e^{0.0209x}$; $r = 0.99$). The AUC was calculated for each cell line by integrating the exponential or logarithmic function of these curves from 0 to 24 hr and by integrating the linear regression equations obtained from 24 to 30 hr (AUC is 66.57 for SK-MG-1 cells and 39.36 for SKI-1 cells).

TABLE 2

Gatase activity in human glioma cells

Cellular protein extracts were reacted with tritiated methyl DNA for 60 min at 37° as described in Materials and Methods. The Gatase activity is expressed as picomoles of radioactivity from [^{3}H]methylguanine in DNA transferred per milligram of cellular protein.

Source of extract	Gatase activity[a]
	pmol/mg of protein
HT29 cells	0.1240 ± 0.027
SK-MG-1 cells	0.0280 ± 0.010
SKI-1 cells	0.0145 ± 0.010[b]

[a] Mean ± standard error for six determinations.
[b] Not statistically different from SK-MG-1 Gatase activity by the two-tailed *t*-test.

Cells. The SKI-1 and SK-MG-1 cells were established from a single untreated human glioma speciman. SKI-1 cells were generously provided by Dr. J. Shapiro, Cornell University, New York. SK-MG-1 cells were a gift from Dr. G. Cairncross, University of Western Ontario, London, Ontario. The human cancer cell line HT29 was kindly supplied by Dr. M. Pollak, Lady Davis Institute for Medical Research, Montreal, Quebec. The cells were grown and maintained in McCoy's 5A medium supplemented with 10% FCS and 4 μg of gentamycin per ml (Shering, Pointe Claire, Quebec, Canada) in a humidified 5% CO_2 atmosphere at 37°. The cells were screened for mycoplasma with the Hoechst Stain Kit, Flow Lab, Mississauga, Ontario, Canada.

Cytotoxicity assay. The cytotoxicity of SarCNU on SK-MG-1 and SKI-1 cells was evaluated in the *in vitro* colony formation assay (CFA) as described elsewhere (2, 4). Incubations of exponentially growing cells with SarCNU were carried out for 1 hr at 37°. After drug treatments, cells were replated in nutrient-rich agarose on semi-solid agar supplemented with nutrients to allow for the formation of colonies. The cytotoxicity is expressed as the percentage of control (ratio of treated colonies to untreated colonies multiplied by 100).

Transport experiments. Tritiated sarcosinamide was utilized to evaluate the transport of SarCNU in SK-MG-1 and SKI-1 cells. SarCNU could not be used in these studies because it is not available

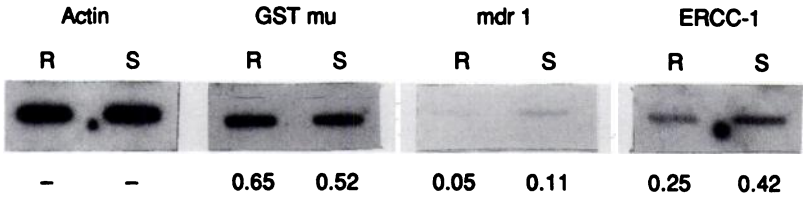

Fig. 7. RNA slot blot analysis of human glioma cells for GST mu, P-glycoprotein, ERCC-1, and actin RNA. Cellular RNA was hybridized to cDNA probes specific for GST mu, mdr1, or actin as described in Materials and Methods. The bands show hybridization signals obtained for 0.625 μg of cellular RNA. The results for the resistant SKI-1 cells (lanes R) and for the sensitive SK-MG-1 (lanes S) are shown. Expression *a* values shown under each lane represent the densitometry measurements for a specific probe divided by the corresponding measurement for actin. The data is expressed relative to actin in order to correct for any differences in the amount of RNA present for each cell line.

in a radiolabeled form. The transport of radiolabeled sarcosinamide was evaluated on monolayers of either SK-MG-1 or SKI-1 cells (10^6 cells/35-mm plate) at 22° or 0° as previously described (14). The cells were assayed for transport in PAG. The measurement of uptake was initiated by the addition of 1 ml of tritiated sarcosinamide plus or minus iso-osmotically adjusted SarCNU in PAG, following a 15- or 30-min preincubation in PAG. At desired time intervals, the radiolabel was rapidly aspirated and the cells were rinsed five times with 2 ml of ice-cold PBS supplemented with 48 mM glycyl glycine. Efflux experiments were initiated by incubating the cells in tritiated sarcosinamide for 10 or 30 min at 0° or 22°. The medium was removed and replaced with an equivalent volume of nonradioactive PAG. The efflux of the radiolabeled sarcosinamide was terminated at indicated times as described above. The cells were solubilized at 60° with 2 ml of 3% sulfosalicylic acid for 10 min, the cell extracts were next heated at 100° for 5 min and then centrifuged at 300 × *g* for 10 min to pellet out cellular debris. Aliquots of the supernatants were dissolved in Scintiverse, and the radioactivity was measured in a Wallac 1217 Rackbeta liquid scintillation spectrophotometer. The number of cells per 35-mm plate was determined by counting cells in 4–6 plates in a Coulter Counter and using the average as the cell number per plate. The mean of the cell number obtained by this method has a standard error of less than 5%. The uptake of sarcosinamide was expressed as nanomoles or picomoles per 10^6 cells.

Measurement of steady-state accumulation of SarCNU. A modified version of the Britton-Marshall method was used to measure the accumulation of SarCNU in the glioma cells (15). Cells in suspension (2×10^6/ml of PAG) were preincubated for 15 min at 37° and then treated with 1 mM SarCNU (the lowest CENU concentration detectable by this assay) for 30 min at 37°. The cells were separated from the medium by centrifugation through versilube oil. The cell pellets, aliquots of the supernatants, and standards of SarCNU were dissolved in 0.5% sulfanilamide in 1 N HCl, incubated at 50° for 45 min and treated with the Britton-Marshall reagent at 22° for 10 min. The absorbance was measured at 540 nm on a Hewlett-Packard 8451A diode array spectrophotometer and micrograms of intracellular or extracellular SarCNU were determined by extrapolation from standard curves. The intracellular water space (ICW) was determined with tritiated water and [*carboxyl*-^{14}C]inulin by centrifugation of the glioma cells through Versilube oil by a previously described method (16). The accumulation of SarCNU in the cells is expressed as the cell to medium ratio which describes the distribution of SarCNU in 1 μl of the ICW and the extracellular medium.

Measurement of DNA cross-links. Suspensions of SK-MG-1 and SKI-1 cells (2×10^6 cells/ml of PAG) were incubated with 0.28 mM of SarCNU (the lowest concentration which produces detectable DNA crosslinks) or in an equivalent volume of vehicle for 1 hr. Drug treatment was terminated by rinsing the cells in PAG. The cells were then suspended in an equivalent volume of PAG and incubated at 37° for selected periods of time to allow for the formation of cross-links. Since the duration of drug-free incubations ranged from 0 to 30 hr, the cells were checked for viability. After 30 hr in suspension, the cells were approximately 98% viable as determined by the trypan blue exclusion test.

The DNA cross-links formed by SarCNU were detected by the ethidium bromide fluorescence assay (17). A 40-μl portion of cells (8×10^6 cells) was lysed in a 200-μl solution containing 4 M NaCl, 0.05 M KH_2PO_4, 0.01 M EDTA, and 0.1% sarcosyl, pH 7.2 The lysates were then treated with 40 μg of heat-inactivated bovine pancreas RNase at 37° for 16 hr followed by treatment with 50 IU of heparin for 20 min at 37°. The reaction mixtures were then added to 3 ml of a solution containing ethidium bromide (10 μg/ml), 20 mM K_2PO_4, and 0.4 mM EDTA, pH 12.1. The fluorescence of treated and control DNA lysates was measured at 22° in a SPF-500C SLM spectrofluorometer at an excitation of 525 nm and an emission of 580 nm. The DNA was denatured at 100° for 5 min followed by rapid cooling at 22°. The fluorescence of the denatured samples was measured and the percentage of cross-linked DNA was evaluated by the formula

$$C_t = \frac{f_t - f_n}{1 - f_n} \times 100$$

where C_t is the percentage of cross-linked DNA in the treated cells, f_t is the ratio of the fluorescence of denatured to native drug-treated DNA lysates, and f_n is the ratio of fluorescence of denatured to native control DNA lysates.

Measurement of Gatase activity. Crude protein extracts were prepared by dissolving SK-MG-1, SKI-1, or HT29 cell pellets in 1 ml/10^8 cells of 50 mM Tris·HCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, pH 7.8 at 4° according to a published procedure (18). The lysates were sonicated six times for 15 sec at 4° and centrifuged at 12,000 × *g* for 2 min at 4°. The supernatants were used for the determination of Gatase activity. The protein extract from the Mer$^+$ HT29 cells was included in the assay as a positive control.

The activity of Gatase was assayed essentially as previously described (19). [^{6}O]methylguanine adducts were introduced into calf thymus DNA by reacting it with [^{3}H]MNU (Du Pont-New England Nuclear; specific activity, 4.4 Ci/mmol) in 0.2 M Tris·HCl, pH 8.0 for 60 min at 37°. The cellular protein extracts (200–600 μg) were mixed with 200 μl of alkylated DNA (4.4 Ci/mmol) in 50 mM Tris·HCl, 1 mM EDTA, 1 mM DTT, pH 7.8 for 60 min at 37°. The reaction was stopped by the addition of 100 μl of 5% TCA and heating the mixture to 80° for 30 min to selectively hydrolyse the DNA. The samples were chilled on ice and filtered by suction onto Whatman GFC filters. The filters were washed once with 5 ml of 5% TCA and twice with 5 ml of ethanol. The radioactivity present in the protein extract was measured by liquid scintillation counting. The activity of Gatase is expressed as picomoles of tritiated methyl group transferred per mg of protein extract.

Measurement of glycosylase activity. The glycosylase activity in the glioma cells was measured according to an established procedure (20). Crude protein extracts of SK-MG-1 and SKI-1 cells were prepared by lysing cell pellets in 100 μl of 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 10 mM DTT, 0.2% Triton X-100 per 10^7 cells at 4°. The lysate was centrifuged at 12,000 × *g* for 10 min at 4° to separate the protein extract in the supernatant. *Micrococcus luteus* DNA (Sigma) was dis-

solved in 10 mM sodium cacodylate, 1 mM EDTA, pH 7.0 and reacted with [^{3}H]dimethylsulfate (Du Pont-New England Nuclear; specific activity, 1.6 Ci/mmol) at 37° for 60 min in the dark. The DNA was precipitated by the addition of 200 μl of 2 M NaCl and 3 volumes of ethanol cooled to −20°. The DNA was dissolved in 2 ml of 10 mM Tris·HCl, 1 mM EDTA, pH 8.0 (TE) and dialysed at 4° against two changes of 500 ml of TE. The reaction was carried out in 100 μl of buffer containing 70 mM Hepes-KOH, 1 mM EDTA, 1 mM DTT, pH 7.8 plus 10^4 cpm [^{3}H]DNA and 2–20 μl of the cellular extract for 60 min at 37°. The DNA was precipitated as above, and the supernatant was assayed for the presence of released DNA adducts by liquid scintillation counting. The glycosylase activity was expressed as the picomoles of radioactivity liberated from the methylated DNA per mg of cellular protein. The DNA adducts were also analyzed by HPLC. After reactions of tritiated methyl DNA with cellular extracts as described above, the supernatants containing the released adducts were lyophilized and dissolved in 6% methanol in deionized water. The samples were injected into a Partisil SCX-10 column and eluted with a gradient of 1–50% 0.1 M ammonium formate in 6% methanol in deionized water at a flow rate of 0.4 ml/min. The radioactivity in the collected fractions was measured by liquid scintillation counting. The glycosylase activity is expressed as the ratio of 3-methyladenine to 3-methyladenine plus 7-methylguanine released ($m^3A/m^3A + m^7G$).

Expression of mdr1, GST mu, and ERCC-1 RNA in glioma cells. Total cellular RNA from SK-MG-1 and SKI-1 cells was isolated by homogenizing the cells in 6 M guanidinium hydrochloride. The RNA samples were denatured in 7× SSC (20× SSC contains 3.0 M NaCl and 0.3 M Na citrate), 7.5% formaldehyde for 15 min at 65°. Serial dilutions (2-fold) of each RNA sample (5–0.078 μg) were prepared in 10× SSC and applied to a nylon membrane (Managraph; Micron Separations Inc., Westborough, MA). The membrane was baked for 2 hr at 70° to fix the RNA. The membrane was prehybridized for 4–12 hr at 68° in 6× SSC, 5× Denhardt's solution, 0.5% $NaDodSO_4$, and 100 μg of denatured salmon sperm DNA per ml. Hybridizations were carried out with ^{32}P-labeled DNA probe at 2×10^6 cpm/ml of the hybridization solution for 36 hr at 68°. The slot blot was washed to a final stringency of 0.1× SSC, 0.1% $NaDodSO_4$ at 65°. Autoradiography was done at 70° on Kodak XAR film with an intensifying screen. The radiolabeled probe was removed by washing the membrane three times (15 min per wash) in 0.1× SSC, 0.5% $NaDodSO_4$ at 95°. The RNA samples were probed with the human mdr1-specific cDNA. Human GST class mu expression was detected by using the insert of plasmid pGTA44, which is complementary to the rat liver GST Yb gene provided by Pickett *et al.* (21), and human ERCC-1 mRNA levels were detected by using the insert of plasmid PE12-12 provided by van Duin (22). The blot was also probed with [^{32}P]actin cDNA to verify the amount of RNA in each sample. The hybridization signals were quantified by densitometry scans of the autoradiograms on an LKB Ultrascan densitometer. Corrections for quantitative differences in RNA samples were obtained by expressing the results as ratios of densitometry measurements for the individual probes to the intensity measured for RNA probed with actin.

GSH. Intracelllular GSH levels were determined by the glutathione reductase assay (23). Glioma cells (10^7) were lysed in 900 μl of H_2O by vortexing for 5 min, and 100 μl of 30% sulfosalicylic acid was then added. The resulting suspensions were left at 0° for 15 min and then centrifuged at 12,000 × *g* for 2 min. Total GSH content was then assayed.

Calculations. The determination of kinetic constants was done by linear regression analysis of Lineweaver-Burke plots or Dixon plots. The AUC was calculated by integrating the linear and exponential components of the C_t versus time plot for each glioma cell line. Statistical analysis was performed by the two-tailed *t*-test.

Results

Cytotoxicity of SarCNU. The responses of the glioma cell lines to treatment with SarCNU are compared in Fig. 1. The concentrations of SarCNU required to reduce the growth of SKI-1 and SK-MG-1 cells to 70% control are 60 and 2.9 μM, respectively. Based on the ratio of these concentrations, the SKI-1 cells are resistant to SarCNU by 20-fold. SKI-1 cells were previously found to be 3-fold more resistant to BCNU than SK-MG-1 cells (2).

Steady state accumulation of SarCNU. The ICWs measured for SK-MG-1 and SKI-1 cells were 0.48 ± 0.07 μl/10^6 cells and 0.54 ± 0.06 μl/10^6 cells, respectively. The difference in these two values is not statistically significant. The cell to medium ratio of SarCNU at steady state was found to be 1.22 ± 0.08 in SK-MG-1 cells and 0.65 ± 0.11 in SKI-1 cells. This 47% decrease in intracellular SarCNU in the resistant cells is statistically significant ($p < 0.005$). The cell to medium ratio of BCNU was found to be 0.68 ± 0.15 in the SKI-1 cells. This value is not statistically different from the cell to medium ratio for SarCNU and the cell to medium ratio of 0.525 ± 0.12 determined for BCNU in SK-MG-1 cells.

Transport of [^{3}H]-*N*-sarcosinamide. Since radiolabeled SarCNU is not available, tritiated sarcosinamide was utilized to examine the transport of SarCNU in SKI-1 cells. The time course of uptake was 0.05 mM tritiated sarcosinamide in SKI-1 cells and SK-MG-1 cells is shown in Fig. 2. The uptake in both cell lines is linear up to 1 min and begins to plateau after 10 min at 22°. The uptake of sarcosinamide was examined for 30 sec in all subsequent experiments to minimize the effects of efflux. The accumulation of this amino acid amide is similar in both cell lines. The transport of sarcosinamide is reduced in each cell line at 0°.

The rate of sarcosinamide uptake at 30 sec in SKI-1 cells is consistent with Michaelis-Menten kinetics, with saturation of the carrier apparent at 4 mM concentration of sarcosinamide. An inverse plot of these parameters is presented in Fig. 3 for sarcosinamide concentrations between 0.05 and 4.0 mM. The kinetic constants obtained for sarcosinamide transport in SKI-1 cells are compared with those previously determined for SK-MG-1 cells in Table 1. At a K_m value of 1.52 mM, the affinity for sarcosinamide in SKI-1 cells is more than 5-fold lower than in SK-MG-1 cells. The V_{max} for sarcosinamide transport in SKI-1 cells represents a 4-fold increase over the maximum uptake velocity observed in the SK-MG-1 cells. The affinity of this transport for SarCNU was estimated in the SKI-1 cells by Dixon plot analysis (24). Fig. 4 shows the effect of increasing external concentrations of SarCNU on the initial transfer of 0.5, 0.75, and 1.0 mM tritiated sarcosinamide. The results confirm that SarCNU is a competitive inhibitor of sarcosinamide transport in SKI-1 cells with an inhibition constant, K_i, of 17.5 mM. This represents a more than 5-fold decrease in the affinity of SarCNU in the SKI-1 cells from that observed in the sensitive SK-MG-1 cells (K_i = 3.26 mM with an extrapolated V_{max} of 0.15 nmol/10^6 cells/min) (5). The extrapolated V_{max} in SKI-1 cells for SarCNU is 0.653 nmol/10^6 cells/min, which is similar to the V_{max} of sarcosinamide obtained in the Lineweaver-Burk plot (Fig. 3). This finding provides further confirmation for purely competitive inhibition of sarcosinamide uptake by SarCNU (24). The efflux of 0.05 mM tritiated sarcosinamide is compared in the two cell lines in Fig. 5. The efflux is linear in both cell lines for 1 min and began to equilibrate after 30 min at 22°, at which time the percentage of intracellular sarcosinamide transferred out of the SKI-1 and SK-MG-1 cells was 54% and 58%, respectively. The efflux of sarcosinamide at 0° was reduced in each cell line; however, it appears to be more temperature dependent in the SKI-1 cells.

Formation of DNA cross-links. The time course of formation of DNA cross-links induced by 0.28 mM SarCNU in the glioma cells is presented in Fig. 6. The maximal percentage of DNA cross-links (C_t) is observed 24 hr after drug treatment in both cell lines. The percentage of DNA cross-links at that time is significantly reduced in the SKI-1 cells to 47% of the C_t measured in the SK-MG-1 cells ($p < 0.05$). In addition, the AUC which was previously shown to correlate with drug-induced cytotoxicity (25), is 41% greater in the SK-MG-1 cells than in the SKI-1 cells.

Gatase activity. Crude cellular protein extracts were assayed for removal of tritiated [^{6}O]methyl from guanine residues in DNA treated with MNU. The results are summarized in Table 2. The HT29 cells are efficient at repairing this DNA adduct, which is in agreement with their previously established Mer$^+$ phenotype (26). By comparison, both glioma cells exhibited low Gatase activity which is comparable with that reported previously for human glioma biopsies (27). The activity of this repair enzyme was not significantly different in the two cell lines.

Expression of mdr1, ERCC-1, and GST mu. The results of slot blot analysis are presented in Fig. 7. The levels of actin RNA are similar in each cell line, confirming that equivalent levels of cellular RNA were compared. Both glioma cell lines express low levels of mdr1, and the expression of this gene is more than 2-fold greater in the sensitive cell line. The expression of GST mu is increased in the resistant cells, as reflected by the ratio of GST to actin, which is 20% higher in the SKI-1 cells. The ratio of ERCC-1 to actin was found to be 41% greater in the sensitive cells, suggesting that excision repair via this group of enzymes is not important for resistance to SarCNU.

Glycosylase activity. Crude protein extracts obtained from SKI-1 and SK-MG-1 cells were assayed for their ability to excise methylated bases from DNA which was modified with tritiated dimethylsulfate. The total glycosylase activities measured in SKI-1 and SK-MG-1 cells were not significantly different (2.4 ± 0.3 pmol/mg of protein and 3.2 ± 0.6 pmol/mg of protein, respectively). The repair of 7-methylguanine and 3-methyladenine was compared following separation of the released adducts on HPLC. The ratios of release of 3-methyladenine to 3-methyladenine plus 7-methylguanine were similar in the sensitive and resistant cells [($m^3A/m^3A + m^7G$) values of 0.74 and 0.78, respectively].

GSH levels. The GSH levels in SK-MG-1 and SKI-1 cells were 2 ± 0.16 and 0.25 ± 0.02 nmol/10^6 cells (means ± standard deviations), respectively.

Discussion

This investigation was carried out to define the mechanisms of resistance to SarCNU in human glioma cells. Particular attention was focused on differences in cellular transport of SarCNU, since unlike other CENUs, this experimental drug is transported into human glioma cells by a process which mediates the uptake of catecholamines (5). Radiolabeled sarcosinamide was used as a probe for the transport of SarCNU in resistant and sensitive cells. The affinity for sarcosinamide uptake was found to be reduced in the resistant cells. Similarly, the observed increase in the K_i value for SarCNU represents a decreased affinity for transport in the resistant cells. Furthermore, the accumulation of 1 mM SarCNU at equilibrium was found to be significantly reduced in the SKI-1 cells, whereas steady state levels of BCNU were similar in both cell lines. The altered affinity in SKI-1 cells would not result in a decreased intracellular concentration of SarCNU because the transport capacity for this process, as reflected by the V_{max}, is also increased in the resistant cells. The velocity of uptake for 1 mM SarCNU can be estimated, given its kinetic parameters, by using the Michaelis-Menten equation (28). At this concentration, the velocity of uptake would be 0.036 nmol/10^6 cells/min in both SKI-1 cells and SK-MG-1 cells. On the basis of these findings, it is concluded that resistance to SarCNU is not related to its influx via the catecholamine carrier in glioma cells. It is conceivable that SarCNU influx into glioma cells occurs by more than one carrier, and, thus, decreased influx by another carrier could be associated with resistance. The possibility that increased efflux contributes to a lower steady-state accumulation of SarCNU in SKI-1 cells was not supported by the finding that the efflux of sarcosinamide was similar in both cell lines. An alternate possibility is that while sarcosinamide and SarCNU share a common pathway for uptake, their efflux may proceed by distinct mechanisms. This was demonstrated with melphalan and methotrexate, two antitumor agents whose influx and efflux proceed by different routes (29, 30). Thus, increased efflux of SarCNU by an alternative system may be involved in the decreased sensitivity of SKI-1 cells to this CENU. Since the expression of mdr1 was 2-fold greater in the sensitive cell line, it is unlikely that the efflux of SarCNU is mediated by P-glycoprotein. However, we determined mRNA levels, and it is conceivable that differences in P-glycoprotein exist that are not concordant with the mRNA levels. Also, a recent report about the overexpression of a novel membrane protein in a cell line resistant to adriamycin and melphalan raises the possibility that other efflux pumps may mediate drug resistance (31).

A time-course study of the formation and removal of DNA cross-links demonstrated a reduction of DNA cross-links in the resistant cell line. This could be secondary to decreased intracellular drug accumulation, decreased formation of DNA cross-links, and/or increased removal of these cross-links. Surprisingly, Gatase activity was similar in both glioma cell lines, suggesting that in this system, this repair activity does not play a role in resistance to SarCNU. The overall glycosylase activity was also similar in both cell lines. The possibility that there may be differences in the removal of a specific DNA adduct was also addressed in this study. HPLC analysis of the major products of glycosylase activity revealed that there is no evidence of preferential removal of 7-methylguanine or 3-methyladenine in the resistant cells. It is possible that there are subtle differences in the removal of other DNA adducts, since *N*-3-methyladenine DNA glycosylase removes several types of methylated purines (32). Differences in removal of CENU-induced DNA alkylation products, possibly by this glycosylase, in sensitive and resistant human glioma cells have been reported (12). Excision repair was not increased in the resistant glioma cells, as reflected by the expression of ERCC-1 RNA in these cell lines. This is in agreement with a previous report which demonstrated that the sensitivity to UV damage was not altered in resistant human glioma cells (12). The lower percentage of DNA cross-links in the resistant SKI-1 cells may be related to the decreased accumulation of SarCNU, since these parameters were reduced by approximately the same amount.

The modest increase in the expression of the isoenzyme GST mu observed in the resistant cells suggests that detoxification of SarCNU is at best a minor contributing factor to resistance. This enzyme was previously shown to contribute to a 3- to 4-fold resistance to BCNU in rat glioma cells (6). The 8-fold greater concentration of GSH in SK-MG-1 cells compared with SKI-1 cells does not support a role for GSH detoxification of SarCNU as a mechanism of resistance. The demonstrated 20-fold resistance to SarCNU in the SKI-1 cells suggests that additional mechanisms mediate the decreased sensitivity to this agent.

This study demonstrates that resistance to SarCNU in SKI-1 cells correlates with decreased drug accumulation and reduced cross-linking of DNA. While detoxification by GST mu is not clearly implicated in this process, it is possible that decreased drug influx via a carrier other than the catecholamine carrier, increased drug efflux, and/or alternative DNA repair are responsible. More definitive studies of SarCNU uptake will be done when radiolabeled SarCNU is available.

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