Extraneuronal Monoamine Transporter Expression and DNA Repair Vis-à-Vis 2-Chloroethyl-3-sarcosinamide-1-nitrosourea Cytotoxicity in Human Tumor Cell Lines[1]

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ABSTRACT

We previously found that 2-chloroethyl-3-sarcosinamide-1-nitrosourea (SarCNU), a new chloroethylnitrosourea analogue presently in phase I clinical trials, is a selective cytotoxin that enters cells via the extraneuronal transporter for monoamine transmitters (EMT). In this study, we assessed whether EMT expression correlates with SarCNU cytotoxicity by determining EMT expression in 23 human tumor cell lines with reverse-transcription PCR. Western blot analysis was used to measure protein levels of the DNA repair genes, *O^6-methylguanine-DNA methyltransferase* (*MGMT*), and *excision repair cross-complementing rodent repair deficiency gene 2* (*ERCC2*). SarCNU cytotoxicity was determined by the sulforhodamine B colorimetric anticancer-drug screening assay and correlated with gene expression. Almost all of the cell lines screened were positive for EMT expression. However, seven cell lines (MGR-1, MGR-2, T98-G, SKI-1, SKNSH, 297, and GBM) expressed low levels of EMT. Although there was no linear correlation between SarCNU cytotoxicity and EMT expression, SarCNU cytotoxicity significantly correlated with ERCC2 protein levels, and MGMT-rich (Mer^+) cell lines (MGMT protein level >0.1) were more resistant to SarCNU than MGMT-poor (Mer^-) cell lines (MGMT protein level <0.1). Moreover, multiple regression analysis indicated that the best correlation with SarCNU cytotoxicity was attainable with EMT plus MGMT and ERCC2 expression. This study suggests that in human tumor cell lines both EMT and DNA repair factors, specifically, MGMT and ERCC2, are important determinants of SarCNU activity. Because EMT is expressed in a wide variety of human tumors, SarCNU should be a more widely effective alternative chemotherapeutic agent.

INTRODUCTION

Nitrosoureas, such as BCNU[3] and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, have long been used as standard chemotherapeutic compounds, specifically for the treatment of central nervous system tumors (1). However, their clinical usefulness is restricted by dose-related toxicity that produces delayed and cumulative myelosuppression (2). In the search for novel analogues with increased antitumor activity and decreased toxicity, SarCNU, a novel derivative of CENU, was found to have interesting characteristics (3). SarCNU contains an amino acid amide group (4), *N*-methylglycinamide, known as sarcosinamide, that allows the drug to enter cells via the EMT, *i.e.*, extraneuronal noradrenaline transporter or $uptake_2$, which has recently been characterized molecularly (5).

Our previous *in vitro* and *in vivo* studies demonstrated that SarCNU was more effective than BCNU against human gliomas (6–9). Using the relatively SarCNU-resistant SKI-1 human glioma cell line and the SarCNU-sensitive SKMG-1 human glioma cell line, we previously demonstrated that SarCNU uptake was more rapid and was saturable in the SKMG-1 cells (10). Furthermore, the characteristic of SarCNU uptake suggested that drug uptake was via the EMT (11). However, the relationship between EMT expression and SarCNU activity in human tumors has of yet to be clarified. In the present study, using reverse transcription-PCR, we determined human EMT expression for 23 human tumor cell lines. Because DNA repair has been related to CENU resistance in human tumors (12, 13), we thus also determined DNA repair protein levels, specifically MGMT and the NER gene *ERCC2*, and correlated these factors to SarCNU cytotoxicity.

MATERIALS AND METHODS

Cell Lines. Twenty-three established human tumor cell lines were used in this study: SF-295, HT-29, ACHN, A-498, 786-0, CAKI-1, SW-620, and SF-767 (National Cancer Institute, Bethesda, MD); T98-G (Dr. D. Yarosh, Applied Genetics

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[3] The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; SarCNU, 2-chloroethyl-3-sarcosinamide-1-nitrosourea; CENU, chloroethylnitrosourea; EMT, extraneuronal transporter for monoamine transmitters; MGMT, O^6-methylguanine-DNA methyltransferase; NER, nucleotide excision repair; FBS, fetal bovine serum; SRB, sulforhodamine B; IC_{90}, inhibitory concentration leading to 90% cell death.

Inc., New York, NY); SKMG-1 and SKMG-4 (Dr. G. Cairncross, University of Western Ontario, Ontario, Canada); SKI-1 (Dr. J. Shapiro, Barrow Neurological Institute, Phoenix, AZ); UWR-7, UW-28, MGR-1, MGR-2, and MGR-3 (Dr. F. Ali-Osman, University of Texas M.D. Anderson Cancer Center, Houston, TX); SKNSH (Dr. E. Shoubridge, Montreal Neurological Institute, Montreal, Canada); MCF-7 (Dr. Gerald Batist, Jewish General Hospital, Montreal, Canada); SHG-44, GBM, and 297 (Dr. Q. Huang, Suzhou Medical College, Suzhou Peoples Republic of China), and HepG2. All cell lines were grown and maintained as cell monolayers in appropriate medium (McCoy's 5A supplemented with 10% FBS, RPMI 1640 supplemented with 5% FBS, or DMEM supplemented with 10% FBS), containing 10 μg/ml gentamicin, in a humidified 5% CO_2 atmosphere at 37°C.

SRB Cytotoxicity Assay. SarCNU cytotoxicity was determined using a modified SRB colorimetric anticancer-drug screening assay (14). Briefly, appropriate amounts of cells were seeded onto 24-well flat-bottomed plates in 0.5 ml of medium. After a 16-h incubation (day 2), the cells were treated with different concentrations of SarCNU (dissolved in 1 mM sodium citrate, pH 4). On day 4, 1.5 ml of medium was added to each well, followed by incubation for 4 more days at 37°C, 5% CO_2. The medium was then aspirated, and cells were fixed onto the plastic substructure by the addition of 1 ml of 10% trichloroacetic acid in 0.9% NaCl and incubation for 1 h at 4°C. The plates were washed five times with water to remove trichloroacetic acid and air-dried for at least 1 h. This was followed by staining with 1 ml of 0.4% SRB in 1% acetic acid for 30 min at room temperature, washing five times with 1% acetic acid to remove unbound dye, and subsequently air-drying. Bound dye was solubilized with 2 ml of 10 mM unbuffered Tris base (pH 10.5). Absorbance was read using a spectrophotometer at 540 nm, and the IC_{90} in μM was obtained by exponential curve fit of the linear portion of the cytotoxicity curve using CA-Cricket Graph III version 1.01 (Computer Associates International, Inc., Islandia, NY).

Determination of EMT Expression. RT-PCR was used to determine EMT expression in the cell lines. Total RNA was extracted using the RNeasy Midi Kit (Qiagen Inc., Valencia, CA) following manufacturer's protocol. The cDNA was synthesized as described previously (13, 14). Primers for the EMT PCR reaction were designed by Steve Rozen and Helen J. Skaletky (1996–1997) using the primer 3 program and synthesized by Canadian Life Technologies (Burlington, ON). The left primer spun from positions 631 to 650 (5′-3′, gcaccaaacttccctgtgtt), and the right primer spun from positions 963 to 944 (5′-3′, agcaatgcgtctcaggatct). The PCR reaction was performed in a total volume of 50 μl consisting of 2.5 μl of 2.5 mM dNTPs, 2 units of DNA polymerase AmpliTaq (Pharmacia), 20 pmol of each primer, and 2 μl of 1st strand cDNA (reverse transcribed from 0.2 μg of total RNA) in 1× PCR buffer (Pharmacia). The PCR cycle comprised 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and elongation at 72°C for 45 s and was run using a PTC-100TM programmable thermal controller (MJ Research Inc., Watertown, MA). β-Actin expression was determined as described previously and was used for normalization (13, 14). The PCR products were run on 1% agarose gel and were quantitated with the Scion Image program using an

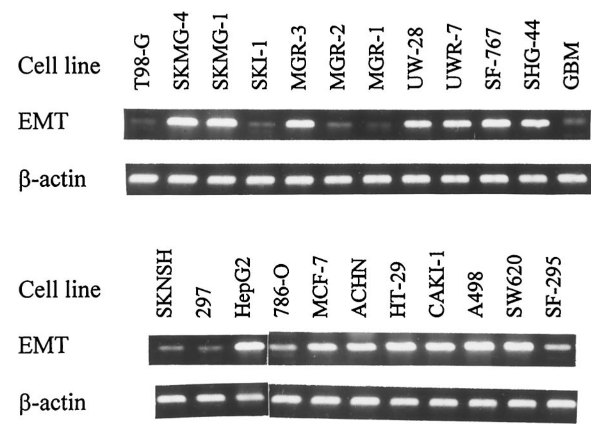

Fig. 1 Human EMT expression in 23 human tumor cell lines. A 1% agarose gel shows the 333-bp *EMT* PCR product and the 315-bp β-*actin* PCR product.

HP ScanJet 5100C Scanner (Hewlett Packard Company, Greeley, Colorado). EMT expression for each cell line was determined by dividing the EMT absorbance value by the β-actin absorbance value. Both EMT and β-actin were in the linear range of PCR amplification. The EMT expression results are the mean of three separate determinations.

Determination of DNA Repair Protein. DNA repair protein MGMT and protein levels coded by one of the NER genes, *ERCC2*, were detected by Western blotting as described previously (15). Similarly, α-tubulin expression was determined. For each cell line, gene expression was normalized by dividing by α-tubulin expression.

Statistical Analysis. The correlation between gene expression and SarCNU cytotoxicity was analyzed using linear regression (StatView 512+ version 1.2). Multiple linear regression analysis that improved *P* were sought. For MGMT expression, the cell lines were divided into two groups, MGMT-rich (Mer^+) and MGMT-poor (Mer^-). The SarCNU cytotoxicity for the Mer^+ and Mer^- groups was analyzed using Student's *t* test.

RESULTS

EMT Expression in Human Tumor Cell Lines. Almost all of the cell lines screened tested positive for EMT expression, although seven cell lines (MGR-1, MGR-2, T98-G, SKI-1, SKNSH, 297, and GBM) were very low EMT expressers (Fig. 1). Eighteen of 23 cell lines with corresponding SarCNU cytotoxicity data were used in linear regression analysis (Table 1). The human hepatoma cell line HepG2 expresses high levels of EMT and thus was used as a positive control.

DNA Repair Protein Expression in Human Tumor Cell Lines. MGMT and ERCC2 protein levels in 18 human tumor cell lines as determined by Western blot analysis are listed in Table 1 (each value represents the mean of at least three separate experiments). The protein levels of MGMT and ERCC2 as determined by Western blot analysis correlates with the mRNA levels ($r = 0.888$; $P = 0.0001$ for MGMT; and $r = 0.674$, $P = 0.0022$ for ERCC2). The mRNA levels for *MGMT* and *ERCC2* were determined by using the same reverse transcription-PCR

Table 1 Screening of a panel of 18 human tumor cell lines for SarCNU cytotoxicity and *ERCC2, MGMT,* and *EMT* gene expression

Cell line (tumor type)	SarCNU cytotoxicity IC_{90} (μM)		Protein level				mRNA level *EMT*[d]	
			ERCC2[b]		MGMT[c]			
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
MCF (B)[a]	218.5	13.5	0.007	0.004	1.000	0	0.384	0.066
SKMG-1 (G)	28.6	2.7	0.004	0.002	0	0	0.740	0.085
T98G (G)	165.2	1.4	0.057	0.026	0.306	0.127	0.227	0.138
SKMG-4 (G)	40.6	2.3	0.061	0.022	0.052	0.025	0.596	0.058
HT-29 (C)	80.7	5.6	0.003	0.003	0.613	0.089	0.883	0.097
SKI-1 (G)	44.8	1.2	0.069	0.029	0.012	0.012	0.053	0.029
SF-295 (G)	46.5	0.8	0.066	0.029	0.037	0.037	0.185	0.057
MGR-3 (G)	266.4	13.5	0.037	0.015	0.022	0.022	0.520	0.080
MGR-2 (G)	36.8	2.3	0.028	0.012	0	0	0.135	0.042
786-0 (R)	36.7	2.3	0.083	0.054	0.023	0.019	0.229	0.071
CAKI-1 (R)	284.8	29.0	0.109	0.044	0.394	0.139	0.823	0.117
ACHN (R)	146.2	11.6	0.208	0.088	0.680	0.011	0.522	0.095
UW28 (G)	62.2	7.6	0.029	0.012	0.134	0.134	0.374	0.096
A498 (R)	451.6	14.4	0.326	0.099	0.130	0.083	1.116	0.064
MGR-1 (G)	72.4	3.5	0.009	0.003	0	0	0.070	0.026
SKNSH (N)	367.7	42.7	1.000	0	0.167	0.094	0.100	0.023
SW-620 (C)	34.0	5.3	0.006	0.004	0.015	0.006	0.670	0.047
UWR-7 (G)	33.4	2.7	0.184	0.067	0	0	0.377	0.091

[a] Tumor types of cell line: B, breast; C, colon; G, glioma; N, neuroblastoma; R, renal.
[b] ERCC2 protein level as determined by Western blot analysis for each cell line was divided by ERCC2 protein level for SKNSH for each experiment. The result is expressed as the mean value of at least three separate experiments.
[c] MGMT protein level determined by Western blot analysis for each cell line was divided by MGMT protein level for MCF-7 for each experiment. The result is expressed as the mean value of at least three separate experiments.
[d] *EMT* mRNA level determined by RT-PCR for each cell line was divided by mRNA level for *HepG2*, which is used as positive control for each experiment. The result is the mean value of three repeated experiments.

Table 2 Correlation between SarCNU cytotoxicity and gene expression in 18 human tumor cell lines

Comparison	*r*	*P*
SarCNU and ERCC2	0.600	0.0084
SarCNU and MGMT	0.292	0.2393
SarCNU and EMT	−0.350	0.1546
SarCNU and MGMT + ERCC2	0.666	0.0124
SarCNU and MGMT + EMT	0.412	0.2483
SarCNU and EMT + MGMT + ERCC2	0.779	0.0051

technique used in this study for determination of EMT expression (data not show).

SarCNU Cytotoxicity. The SarCNU cytotoxicity in 18 human tumor cell lines was expressed as the IC_{90} (μM). Each value corresponds to the mean of at least three separate experiments (Table 1).

Comparison of EMT Expression with SarCNU Cytotoxicity in Tumor Cell Lines. There was no significant linear correlation between SarCNU cytotoxicity and EMT expression in 18 tumor cell lines. We previously have demonstrated that both MGMT and ERCC2 contribute to BCNU drug resistance in human tumors (13, 14, 16, 17). In the present study, SarCNU cytotoxicity significantly correlated with ERCC2 protein levels but failed to correlate with MGMT protein levels (Table 2). However, Mer^+ cell lines (MGMT protein level >0.1) were more resistant to SarCNU than Mer^- cell lines (MGMT protein level <0.1; IC_{90}: Mer^+, 222.1 ± 45.5 μM; Mer^-, 63.9 ± 22.8 μM; $t = 3.15$; $P = 0.003$). Moreover, EMT and MGMT improved the correlation between SarCNU cytotoxicity and ERCC2, and the best correlation was generated using all three factors (Table 2).

DISCUSSION

EMT uptake activity has been determined for four human tumor cell lines: SKI-1, SF-295, SKMG-1, and CAKI-1 (5, 10, 11);[4] the activity levels were nondetectable, borderline, high, and high, respectively. Unfortunately, at present we are not able to determine protein levels for EMT because the antibody is not yet available. However, the PCR result of *EMT* expression in these cell lines as determined in this study correlates with their pump activity. Grundemann *et al.* (5) demonstrated that *EMT* cDNA transfected into the EMT-negative cell line 293 increased the uptake of known substrates of EMT.

Our previous *in vitro* and *in vivo* studies demonstrated that SarCNU was more effective than BCNU in human tumors (6–9). Using transport studies with radiolabeled SarCNU, we demonstrated that the uptake and accumulation of SarCNU in SKMG-1 cells are significantly greater than in SKI-1 cells, which corresponded with their EMT expression and correlated with the increased sensitivity of SK-MG-1 cells to SarCNU compared with SKI-1 cells (10, 11). SarCNU antitumor activity

[4] Zhong-Ping Chen and Lawrence C. Panasci, unpublished data.

was also evaluated *in vivo* with the human glioma xenografts, SF-295, U-251, and SHG-44 (8, 9). SarCNU was more effective than BCNU against these tumors, which have been confirmed to be EMT positive, suggesting that EMT is important in the *in vivo* response to SarCNU.

In the present investigation, we did not find a linear correlation between SarCNU cytotoxicity and EMT expression. This suggests that EMT expression is not the dominant factor in SarCNU cytotoxicity. However, multiple regression analysis demonstrated that the best correlation was generated with EMT expression plus MGMT and ERCC2 expression, indicating that both DNA repair (MGMT and ERCC2) and EMT are important in determining the sensitivity to SarCNU. It has been documented by both laboratory and clinical evidence that MGMT plays an important role in CENU drug resistance (12, 18–21). We have also demonstrated that NER, specifically ERCC2, expression correlates with CENU resistance in human tumor cell lines (13, 14, 16). In the present study, we found a significant correlation between ERCC2 protein levels and SarCNU cytotoxicity, and Mer$^+$ cell lines were more resistant to SarCNU than Mer$^-$ cell lines. It thus seems that the absence of a linear correlation between SarCNU cytotoxicity and EMT expression in these human tumor cell lines may be due, at least in part, to the presence of DNA repair factors such as MGMT and ERCC2. Thus, whereas MGMT and ERCC2 decrease SarCNU activity by repairing damaged DNA, the presence of EMT appears to increase SarCNU activity. This suggests that EMT is an important determinant of SarCNU activity, possibly by enhanced cellular uptake via EMT and thus higher intracellular SarCNU levels.

The EMT exists in various cells, including glia cells of the human central nervous system and some tumor cells (5, 22). In this panel of 23 human tumor cell lines of different origin, the majority (~70%) are EMT-high expressers. We recently also examined 30 primary human brain tumor specimens for EMT expression and found that only 3 samples have no detectable EMT expression.[5] Because the majority of human tumors express EMT, SarCNU should be a more widely effective alternative chemotherapeutic agent. The presence of the EMT could serve as a marker to identify cancer patients who may be potential responders to SarCNU in the clinic. This bears direct clinical relevance because SarCNU is in phase I clinical trials.

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