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

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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, O6-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 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 uptake2, 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-O, 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, O6-methylguanine-DNA methyltransferase; NER, nucleotide excision repair; FBS, fetal bovine serum; SRB, sulforhodamine B; IC50, 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. G. 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 gentamycin, in a humidified 5% CO₂ atmosphere at 37°C.

**SBR Cytoxicity Assay.** SarCNU cytotoxicity was determined using a modified SBR 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% CO₂. 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% SBR 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 IC₅₀ 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. Skalety (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’, gcaccaaacttccctgcgtgtt), 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). -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 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

![Fig. 1](image-url) 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.
Correlation between SarCNU cytotoxicity and gene expression in 18 human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line (tumor type)</th>
<th>SarCNU cytotoxicity IC\textsubscript{90} ((\mu)M)</th>
<th>ERCC2\textsuperscript{b} Protein level</th>
<th>MGMT\textsuperscript{c} Protein level</th>
<th>mRNA level \textsuperscript{d} EMT \textsuperscript{d} mRNA level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>MCF (B)\textsuperscript{e}</td>
<td>218.5</td>
<td>13.5</td>
<td>0.007</td>
<td>0.004</td>
</tr>
<tr>
<td>SKMG-1 (G)</td>
<td>28.6</td>
<td>2.7</td>
<td>0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>T98G (G)</td>
<td>165.2</td>
<td>1.4</td>
<td>0.057</td>
<td>0.026</td>
</tr>
<tr>
<td>SKMG-4 (G)</td>
<td>40.6</td>
<td>2.3</td>
<td>0.061</td>
<td>0.022</td>
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<tr>
<td>HT-29 (C)</td>
<td>80.7</td>
<td>5.6</td>
<td>0.003</td>
<td>0.003</td>
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<tr>
<td>SKI-1 (G)</td>
<td>44.8</td>
<td>1.2</td>
<td>0.069</td>
<td>0.029</td>
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<td>SF-295 (G)</td>
<td>46.5</td>
<td>0.8</td>
<td>0.066</td>
<td>0.029</td>
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<tr>
<td>MGR-3 (G)</td>
<td>266.4</td>
<td>13.5</td>
<td>0.037</td>
<td>0.015</td>
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<tr>
<td>MGR-2 (G)</td>
<td>36.8</td>
<td>2.3</td>
<td>0.028</td>
<td>0.012</td>
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<tr>
<td>786-0 (R)</td>
<td>36.7</td>
<td>2.3</td>
<td>0.083</td>
<td>0.054</td>
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<tr>
<td>Caki-1 (R)</td>
<td>284.8</td>
<td>29.0</td>
<td>0.109</td>
<td>0.044</td>
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<td>ACHN (R)</td>
<td>146.2</td>
<td>11.6</td>
<td>0.208</td>
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<tr>
<td>UW28 (G)</td>
<td>62.2</td>
<td>7.6</td>
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<tr>
<td>A498 (R)</td>
<td>451.6</td>
<td>14.4</td>
<td>0.326</td>
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<td>MGR-1 (G)</td>
<td>72.4</td>
<td>3.5</td>
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<tr>
<td>SKNSH (N)</td>
<td>367.7</td>
<td>42.7</td>
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<tr>
<td>SW-620 (C)</td>
<td>34.0</td>
<td>5.3</td>
<td>0.006</td>
<td>0.004</td>
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<tr>
<td>UWR-7 (G)</td>
<td>33.4</td>
<td>2.7</td>
<td>0.184</td>
<td>0.067</td>
</tr>
</tbody>
</table>

\(t = 3.15; P = 0.003\). 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);\textsuperscript{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

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\(a\) 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 SKNHS 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.

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

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

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**4 Zhong-Ping Chen and Lawrence C. Panasci, unpublished data.**
was also evaluated in vivo with the human glioma xenographs, 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 expression was more resistant to SarCNU than Mer cells. 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 glial 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. 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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REFERENCES


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