Both Extraneuronal Monoamine Transporter and $O^6$-Methylguanine-DNA Methyltransferase Expression Influence the Antitumor Efficacy of 2-Chloroethyl-3-sarcosinamide-1-nitrosourea in Human Tumor Xenografts

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ABSTRACT

We previously have found that 2-chloroethyl-3-sarcosinamide-1-nitrosourea (SarCNU) is a selective cytotoxin that enters cells via the extraneuronal transporter for monoamine transmitters (EMT). Both in vitro and in vivo studies demonstrated that SarCNU was more effective than BCNU against human gliomas. To clarify whether EMT expression correlates with antitumor efficacy of SarCNU, we determined human EMT (EMTh) and $O^6$-methylguanine-DNA methyltransferase (MGMT) expression in nine human xenograft models using semiquantitative reverse-transcription polymerase chain reaction. These results were compared with the antitumor effects of SarCNU and the standard chloroethylnitrosourea antitumor agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). There was no significant correlation between EMTh expression and antitumor efficacy of SarCNU or BCNU. Also, there was no significant correlation between MGMT expression and SarCNU efficacy. However, a significant correlation was found between MGMT expression and BCNU antitumor efficacy. Interestingly, multiple regression analysis demonstrated a significant correlation between SarCNU efficacy and EMTh plus MGMT expression, whereas there was no correlation between BCNU efficacy and MGMT expression. Thus, the absence of a linear correlation between SarCNU efficacy and EMTh expression appears to be due, at least in part, to the presence of DNA repair, specifically, MGMT, in these xenograft models. These studies suggest that MGMT expression alone correlates with BCNU activity, whereas both EMTh and MGMT expression are important determinants of SarCNU activity against human tumor xenograft models. SarCNU is in clinical trials and these results may have important clinical implications.

Nitrosoureas, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), have long been used as the standard chemotherapeutic compounds, specifically for the treatment of central nervous system tumors (Lesser and Grossman, 1994). Unfortunately, clinical use of these drugs is restricted by dose-related toxicity, producing delayed and cumulative myelosuppression (Carter et al., 1972). In search for novel analogs with increased antitumor activity and decreased toxicity, 2-chloroethyl-3-sarcosinamide-1-nitrosourea (SarCNU) was found to have interesting characteristics (Panasci et al., 1985, 1996).

SarCNU contains an amino acid amide group (Suami et al., 1982), N-methylglycinamide, known as sarcosinamide, which allows the drug to enter cells via the extraneuronal transporter for monoamine transmitters (EMT). The human EMT (EMTh) has recently been molecularly characterized (Grundemann et al., 1998). Our previous in vitro and in vivo studies demonstrated that SarCNU was more effective than BCNU against human gliomas (Skalski et al., 1988; Marcantonio et al., 1997; Chen et al., 1999a). Using the relatively SarCNU-resistant SKI-1 human glioma cell line and the SarCNU-sensitive SKMG-1 cell line, we demonstrated that SarCNU uptake was more rapid and was saturable in the SKMG-1 cells (Noé et al., 1994). Furthermore, the characteristics of SarCNU uptake suggested that drug uptake was via the EMT (Noé et al., 1996). Using reverse-transcription polymerase chain reaction (RT-PCR), we have determined that SKMG-1 is

ABBREVIATIONS: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; SarCNU, 2-chloroethyl-3-sarcosinamide-1-nitrosourea; EMT, extraneuronal transporter for monoamine transmitter; EMTh, human EMT; RT-PCR, reverse-transcription polymerase chain reaction; MGMT, $O^6$-methylguanine-DNA methyltransferase; bp, base pair(s).
EMT-rich, whereas SKI-1 is EMT-poor with approximately a 14-fold difference (Chen et al., 1999b). We previously evaluated the antitumor activity of SarCNU with the human glioma xenografts SF-295, U-251, and SHG-44 (Marcantonio et al., 1997; Chen et al., 1999a). SarCNU was more effective than BCNU against these tumors and all these tumor cell lines were EMT-positive (Chen et al., 1999b), suggesting that EMT may also be important in the in vivo response to SarCNU.

To clarify whether EMT expression contributes to the antitumor efficacy of SarCNU, we presently used RT-PCR to determine EMTh expression for nine human tumor xenograft models and correlated the expression with antitumor efficacy of SarCNU in these models.

**Materials and Methods**

**Xenograft Models.** Human tumor xenograft models using athymic nude nu/nu (National Cancer Research) for evaluation of antitumor efficacy of SarCNU has been previously described (Marcantonio et al., 1997). All animal studies were conducted in American Association for the Accreditation of Laboratory Animal Care-approved facilities following United States Public Health Service guidelines. The animals bearing human tumors were treated with the maximally tolerated dosage of SarCNU or BCNU, which results in no drug-related death. The dose schedules in each xenograft model for SarCNU and BCNU treatment were near optimal (Table 1). The antitumor effects were evaluated by calculating the changes in tumor size (T/C%) twice weekly as previously described (Tomayko and Reynolds, 1989; Marcantonio et al., 1997). A T/C% < 40 is considered active as established based on the in vivo efficacy evaluations of conventional chemotherapeutic agents. The different dosage regimens reflect an attempt to optimize the therapeutic index. These different dosage regimens do not appear to significantly alter the therapeutic index of these agents. Tumor specimens of each xenograft model (untreated animals) were used to determine gene expression.

**Determination of Gene Expression in the Tumor Specimens.** Because there is no antibody to measure EMTh protein levels, mRNA expression was determined. Total RNA was extracted from the tumor specimens obtained from xenografts using the RNaseasy Midi kit (Qiagen, Valencia, CA) following the manufacturer’s protocol, and used to synthesize cDNA. The EMTh expression was determined using RT-PCR as described (Chen et al., 1999b). Briefly, primers were designed using the primer 3 program (Steve Rozen, Helen J. Skaletky, Whitehead Institute for Biomedical Research, Cambridge, MA, 1996–1997) and synthesized by Canadian Life Technologies (Burlington, Ontario, Canada). The PCR reaction was performed in a total volume of 50 ml consisting of 2.5 ml of 2.5 mM dNTPs, 2 units of DNA polymerase Ampli Taq (Pharmacia, Montreal, Canada), 20 pmol of each primer, and 2 ml of cDNA preparation (synthesized from 0.2 mg 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 in a PTC-100TM programmable thermal controller (MJ Research Inc., Watertown, MA). We also measured MGMT mRNA by RT-PCR using a described technique (Mineura et al., 1996). Briefly, the PCR reaction volume of 50 ml consists of 2.5 ml of 2.5 mM dNTPs, 1.5 units of DNA polymerase Ampli Taq (Pharmacia), 100 pmol of each primer, and 10 ml of cDNA preparation (synthesized from 1 mg of total RNA) in 1× PCR buffer (Pharmacia). The PCR products of MGMT and EMTh expression were both within the linear range of PCR amplification. β-Actin expression was measured as previously described and was used for normalization (Chen et al., 1997a). To confirm that our RT-PCR expression is semiquantitation, the RT-PCR determination of MGMT mRNA was compared with MGMT protein levels and activity in 16 cell lines. There was an excellent correlation of mRNA expression and either protein levels (r = 0.89, p = 0.0012) or MGMT activity (r = 0.772, p = 0.0012), indicating that RT-PCR determination of MGMT mRNA is indicative of the MGMT status (Z. P. Chen and L. Panasci, unpublished data).

**Statistical Analysis.** The optimal changes in tumor size T/C% from the xenograft model receiving different treatments were correlated with gene expressions using multiple linear regression (Microsoft Excel 97).

**Results**

**Antitumor Efficacy of SarCNU and BCNU.** In the majority of tumor-bearing animal models, treatment with SarCNU was effective in reducing tumor size. However, in two-tumor models (HT-29 and RXF-393), treatment with SarCNU resulted in an optimal T/C% greater than 40, which is considered as ineffective. There were four (of nine) xenograft models where treatment with SarCNU resulted in one of six, six of six, six of six, and nine of ten tumor-free survival (22 of 66 animals). Although BCNU treatment was also effective in most of the models, in only two models did BCNU treatment result in tumor-free animals (Table 2).

**EMTh and MGMT Expression in Xenograft Tumors.** All of the samples had detectable EMTh expression with a range of 4.5-fold difference (Fig. 1; Table 2). However, MGMT was only detected in four tumor types (Fig. 1; Table 2). The MGMT results obtained by RT-PCR correlate with previously published MGMT activity and protein levels in six of nine tumor cell lines (Ostrowski et al., 1991; Chen et al., 1997b, 1999b).

**Comparison of Antitumor Effect of SarCNU or BCNU with Gene Expression.** The optimal T/C% was used to quantify antitumor efficacy following treatment, and compared with gene expression. There was no significant correlation between EMTh expression and antitumor efficacy of SarCNU in these models.

**TABLE 1**

Near-optimal treatment regimens of SarCNU and BCNU used for efficacy testing in human tumor xenograft models

<table>
<thead>
<tr>
<th>Xenograft Model</th>
<th>Near-Optimal Dosage Schedule</th>
<th>BCNU Dosage/Units Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SarCNU</td>
<td>BCNU</td>
</tr>
<tr>
<td></td>
<td>mg/kg/dose</td>
<td>Schedule, Route</td>
</tr>
<tr>
<td>HT-29</td>
<td>200</td>
<td>Q7D × 2, i.v.</td>
</tr>
<tr>
<td>SW620</td>
<td>133.4</td>
<td>Q4D × 3, i.v.</td>
</tr>
<tr>
<td>CAI-1</td>
<td>120</td>
<td>QD × 5, i.p.</td>
</tr>
<tr>
<td>A498</td>
<td>200</td>
<td>Q4D × 3, i.p.</td>
</tr>
<tr>
<td>RXF-393</td>
<td>54</td>
<td>QD × 5, i.p.</td>
</tr>
<tr>
<td>SF-295</td>
<td>167</td>
<td>Q4D × 3, i.v.</td>
</tr>
<tr>
<td>U251</td>
<td>120</td>
<td>QD × 5, i.p.</td>
</tr>
<tr>
<td>SNB-75</td>
<td>120</td>
<td>QD × 5, i.p.</td>
</tr>
</tbody>
</table>

* Specifies the type of tumor cell line: colon, HT-29 and SW620; renal, CAI-1, A498, and RXF-393; central nervous system, SF-295, U251, and SNB-75; lung, NCI-H522.
TABLE 2

EMTh and MGMT expression vis-à-vis anticancer efficacy of SarCNU and BCNU in human tumor xenograft models

<table>
<thead>
<tr>
<th>Xenograft Model</th>
<th>RT-PCR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SarCNU Treatment</th>
<th>BCNU Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MGMT</td>
<td>EMTh</td>
<td>Tumor-Free Animal&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT-29</td>
<td>1.331</td>
<td>0.457</td>
<td>0/10</td>
</tr>
<tr>
<td>SW620</td>
<td>0</td>
<td>0.558</td>
<td>0/10</td>
</tr>
<tr>
<td>CAKI-1</td>
<td>0.409</td>
<td>0.386</td>
<td>0/6</td>
</tr>
<tr>
<td>A498</td>
<td>0</td>
<td>0.540</td>
<td>1/6</td>
</tr>
<tr>
<td>RXF-393</td>
<td>0.515</td>
<td>0.120</td>
<td>0/6</td>
</tr>
<tr>
<td>SF-295</td>
<td>0</td>
<td>0.150</td>
<td>9/10</td>
</tr>
<tr>
<td>U251</td>
<td>0</td>
<td>0.190</td>
<td>6/6</td>
</tr>
<tr>
<td>SNB-75</td>
<td>0</td>
<td>0.385</td>
<td>6/6</td>
</tr>
<tr>
<td>NCI-H522</td>
<td>0.524</td>
<td>0.307</td>
<td>0/6</td>
</tr>
</tbody>
</table>

<sup>a</sup> MGMT and EMTh expression were determined by RT-PCR and quantitated by densitometry as described under Materials and Methods. The results were normalized by β-actin expression and were the mean value of three separate experiments.

<sup>b</sup> Tumor free refers that by the final observation day, the tumor regressed below the measurable size (32 mg).

<sup>c</sup> %TC = (ΔT/ΔC) × 100, where ΔT > 0 or ΔT = C, where ΔT > 0, ΔT/ΔC represents change in tumor weight for treated (T) and control (C) group, respectively.

<sup>d</sup> Ti is the median tumor weight at the start of treatment. The day on which this optimal (minimum) TC occurred is shown in parentheses.

**Discussion**

Our previous in vitro studies demonstrated that SarCNU was more effective than BCNU against human gliomas (Pansaci et al., 1985; Skalski et al., 1988). Using transport studies with radiolabeled SarCNU we have demonstrated that the uptake of SarCNU in SKMG-1 cells was more rapid and there was a greater accumulation of SarCNU in SKMG-1 cells compared with SKI-1 cells. This corresponded to the cytotoxicity results, i.e., SKMG-1 cells were more sensitive to SarCNU (Noe et al., 1994, 1996). Using RT-PCR we have confirmed that SKI-1 cells expressed EMTh much less than SKMG-1 cells (Chen et al., 1999b), supporting that the differential cytotoxicity to SarCNU in these cell lines is due to the presence of the EMTh in SKMG-1 cells. In the present in vivo study, we did not find a correlation between SarCNU antitumor effect and EMTh expression alone, but instead a significant correlation was found with EMTh expression and MGMT expression together. The absence of a linear correlation between SarCNU cytotoxicity and EMTh expression appears to be due, at least in part, to the presence of DNA repair factors such as MGMT. Thus, although the expression of MGMT will diminish the activity of both SarCNU and BCNU, the expression of EMTh will increase the activity of SarCNU. This suggests that the expression of both EMTh and MGMT is an important factor in SarCNU activity, possibly by increasing intracellular SarCNU levels due to enhanced cellular uptake via EMTh.

It has been documented that MGMT plays an important role in BCNU drug resistance (Brent et al., 1985; Mitchell et al., 1992; Phillips et al., 1997). This study confirms the importance of MGMT in chloroethylnitrosourea antitumor activity. There was no correlation of BCNU efficacy with MGMT expression, suggesting that EMTh expression does not play a role in BCNU cytotoxicity. This is in agreement with the fact that BCNU enters cells via passive diffusion and thus the presence of EMTh should have no effect on its cytotoxic effects (Begleiter et al., 1977). Thus, for tumors with similar levels of such DNA repair proteins, the presence of the EMTh may be a determining factor in responsiveness to SarCNU but not to BCNU.

Recently, we examined a panel of 23 human tumor cell
lines of different origin for EMTh expression. Although most of the cell lines were EMTh-positive, seven cell lines were EMTh-poor (Chen et al., 1999b). Assuming that human tumor cell lines are reflective of the clinical situation, SarCNU should prove to be a more useful alternative chemotherapeutic agent than BCNU for treatment of human tumors, including gliomas. The presence of the EMTh transporter could be used to identify cancer patients who may be potential responders to SarCNU in the clinic. This bears direct clinical relevance since SarCNU is in phase I clinical trials.

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References


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