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Transport of (2-Chloroethyl)-3-sarcosinamide-1-nitrosourea in the Human Glioma Cell Line SK-MG-1 Is Mediated by an Epinephrine-Sensitive Carrier System

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SUMMARY

The transport of (2-chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU), an experimental anticancer compound, was investigated in the human glioma cell line SK-MG-1. The transport of [^{3}H]SarCNU was examined in suspension. The uptake of [^{3}H]SarCNU was found to be temperature dependent, with influx being linear to 4 sec at 37°. Equilibrium was reached after 1 min at 22° and 37°, with accumulation slightly above unity. SarCNU was not significantly metabolized in the cells after a 60-min incubation at 37°, as shown by thin layer chromatography. At 37°, uptake of [^{3}H]SarCNU was found to be saturable, sodium independent, and energy independent. Previous work demonstrated that SarCNU was able to inhibit the uptake of sarcosinamide, which is transported by the catecholamine uptake 2 system. This catecholamine system mediates the physiological transport of epinephrine. Epinephrine was able to significantly inhibit the uptake of [^{3}H]SarCNU, at a concentration of 50 μM, by 40%. Additionally, several amino acids were unable to inhibit the uptake of SarCNU. The initial rate of SarCNU influx is mediated by both facilitated and nonfacilitated diffusion. The nonfacilitated diffusion rate could be estimated from the linear concentration dependence of the residual influx rate for SarCNU, which was not inhibited by the presence of excess co-permeant (epinephrine). Dixon plot analysis, corrected for nonfacilitated diffusion of SarCNU, revealed that epinephrine inhibited the uptake of SarCNU competitively, with a K_i of 163 ± 15 μM, a value similar to the K_m value for epinephrine influx in SK-MG-1 cells. Additionally, after appropriate corrections for nonfacilitated diffusion in the influx rates observed for SarCNU, it was revealed that SarCNU influx obeyed Michaelis-Menten kinetics over a 200-fold range of concentrations, with a K_m of 2.39 ± 0.37 mM and a V_{max} of 236 ± 53 pmol/μl of intracellular water/sec. Metabolic poisons (2,4-dinitrophenol, iodoacetate, NaCN, NaF, or ouabain) were unable to inhibit the influx of SarCNU, suggesting that the carrier-mediated uptake of SarCNU is energy independent and mediated by facilitated diffusion. These findings indicate that SarCNU uptake in SK-MG-1 cells is mediated both by nonfacilitated diffusion and by facilitated diffusion via the catecholamine uptake 2 carrier system. SarCNU is the first chloroethylnitrosourea that has been demonstrated to have carrier-mediated uptake. Moreover, this carrier-mediated uptake may play a role in the increased cytotoxicity of SarCNU against gliomas, compared with that of 1,3-bis(2-chloroethyl)-1-nitrosourea, which enters cells primarily by passive diffusion.

SarCNU, an experimental anticancer compound, is a CENU analogue that contains the amino acid amide group *N*-methylglycinamide, known as sarcosinamide (1). Previously, SarCNU has been shown to be more active than the clinically available CENU analogue BCNU in primary glioma cells and glioma cell lines *in vitro* (2) and in human glioma cells intracerebrally implanted into nude mice (3). In addition, SarCNU is less toxic than BCNU in mice (1) and less myelotoxic in the *in vitro* colony-forming unit-C assay with normal human bone marrow (4). Furthermore, SarCNU is more cytotoxic *in vitro* at its theoretical PPC of 68 μM, compared with BCNU at its clinically achievable PPC of 9 μM (2). The presence of the sarcosinamide carrier moiety in SarCNU suggests that transport into cells may be altered, compared with that of BCNU and other clinically available CENUs, which enter cells via passive diffusion (5).

Previously, the hypothesis that SarCNU may not enter cells purely by passive diffusion was tested indirectly by using a modified version of cytotoxicity as an indicator of transport of an antitumor agent (6, 7). The cytotoxicity of SarCNU in the human glioma cell line SK-MG-1 was not reduced in media that contained excess amounts of amino acids. However, the presence of excess sarcosinamide during *in vitro* cytotoxicity assays significantly decreased the cytotoxicity of SarCNU but

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ABBREVIATIONS: SarCNU, (2-chloroethyl)-3-sarcosinamide-1-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CENU, chloroethylnitrosourea; ICW, intracellular water space; PAG, Dulbecco's incomplete phosphate-buffered saline supplemented with 0.7% bovine serum albumin (fraction V), 0.25% dextrose, and 0.001% phenol red, pH 7.4; PPC, peak plasma concentration.

not BCNU, suggesting that SarCNU may not enter cells purely by passive diffusion (7). Additionally, kinetic analysis of sarcosinamide influx was used as an indirect measure of SarCNU uptake. Sarcosinamide was used because it was expected to be the native substrate for the transporter of SarCNU and was thought to be more appropriate for kinetic analysis. Influx of sarcosinamide was demonstrated to be saturable, *trans*-stimulatable, temperature dependent, sodium independent, and energy independent. Kinetic analysis revealed a K_m and V_{max} for sarcosinamide of approximately 280 μM and 154 pmol/10^6 cells/min, respectively, in SK-MG-1 cells. Furthermore, Dixon plot analysis showed that SarCNU was able to competitively inhibit the uptake of sarcosinamide with a K_i of 3.26 mM. Analysis of several physiological inhibitors revealed that epinephrine was able to inhibit the uptake of sarcosinamide. Importantly, kinetic analysis, including Dixon plot analysis, of epinephrine and sarcosinamide uptake demonstrated that the two compounds have similar affinities for a common catecholamine carrier. These studies have suggested the existence of a catecholamine uptake 2 carrier system in the glioma cell line SK-MG-1 that may accommodate SarCNU (8).

To properly characterize SarCNU uptake, [^{3}H]SarCNU has been obtained. The effects of various physiological inhibitors, including epinephrine, metabolic inhibitors, and temperature, were examined at [^{3}H]SarCNU concentrations in the range of the theoretical PPC of SarCNU in SK-MG-1 cells. The method of Domin *et al.* (9) was used to estimate the diffusional component of SarCNU accumulation by using an excess amount of the co-permeant epinephrine. The kinetic parameters (K_m and V_{max}) of SarCNU were defined after correction of SarCNU accumulation for diffusion. Additionally, the method of Dixon (10) was used to determine whether epinephrine inhibits the transport of SarCNU in a competitive fashion and to obtain the inhibition constant, K_i, for epinephrine.

Materials and Methods

Drugs. SarCNU (NSC 364432) was a gift from Dr. T. Suami, Keio University (Tokyo, Japan). It was dissolved in 0.001 M citrate buffer, pH 4.0, aliquoted, and stored at −20°.

Materials. (±)-Epinephrine HCl and all unlabeled amino acids were obtained from Sigma Chemical Co. [^{3}H]SarCNU (342 mCi/mmol) was prepared by Amersham Laboratories (Buckinghamshire, England), using the technique described by Suami *et al.* (1). Briefly, [^{3}H]sarcosinamide was prepared by using a catalyzed exchange reaction with tritiated water of high specific activity, and the crude preparation was purified by paper chromatography. The [^{3}H]sarcosinamide was then reacted with 2-chloroethyl isocyanate in dry methanol and the resultant crude *N*-carbamoyl intermediate was reacted with sodium nitrate in 99% formic acid to produce [^{3}H]SarCNU, which was purified by high performance liquid chromatography. The radiochemical purity was >99%, as determined using thin layer chromatography on no. 13179 silica gel plates (Kodak) with a solvent system of butan-1-ol/4.0 M sodium acetate, pH 4.0/water (4:2:1) (R_F, 0.81). [*carboxyl*-^{14}C]Inulin (3.2 mCi/mmol), tritiated water (1 Ci/ml), and bovine serum albumin (fraction V, powder; low salt and salt-free fractions) were purchased from ICN. McCoy's 5A modified medium, fetal calf serum, and Dulbecco's phosphate-buffered saline were supplied by Canadian Life Technologies (GIBCO, Montreal, Canada). Versilube F-50 silicone oil was purchased from Nessa Products (Montreal, Canada). Dextrose and NaCl were obtained from Fisher Laboratories. Tris base was purchased from Sigma. Metabolic poisons (2,4-dinitrophenol, iodoacetic acid sodium salt, NaCN, NaF, and ouabain octahydrate) were purchased from Anachemia.

Cell culture. SK-MG-1 cells were established from an untreated human glioma specimen and were a gift from Dr. G. Cairncross (University of Western Ontario, London, Ontario, Canada). The cells were grown and maintained in McCoy's 5A medium supplemented with 10% fetal calf serum and 10 μg/ml gentamycin (Schering, Pointe Claire, Quebec, Canada), in a humidified 5% CO_2 atmosphere at 37°. The cells were found to be free of *Mycoplasma* with the Hoechst stain kit (Flow Laboratories, Mississauga, Ontario, Canada). Confluent monolayers of cells were washed once with PAG and harvested with a rubber policeman. Cell suspensions were centrifuged at 300 × *g* for 5 min, washed twice in PAG, and resuspended in PAG to either 4 × 10^6 or 2 × 10^6cells/ml, as needed.

Transport experiments. Two methods were used to assay the uptake of [^{3}H]SarCNU in SK-MG-1 cells. In the first method, transport of [^{3}H]SarCNU, at a 50 μM concentration (specific activity, 6.84 μCi/μmol), was assayed on confluent monolayers of SK-MG-1 cells (1 × 10^6 cells/35-mm plate) at 22°, as described previously (8, 11). In the second method, [^{3}H]SarCNU at a 50 μM concentration (specific activity, 6.84 μCi/μmol) was used to assay transport at 0°, 22°, and 37° in SK-MG-1 cells, in suspension, with a modified version of "oil-stop" methodology (6, 8, 12). The papaverine-stop methodology used for purine nucleobase uptake analysis, which allows for earlier time points, was not satisfactory because the method did not prevent diffusion of SarCNU (9). Assays were performed in 1.5-ml microcentrifuge tubes with PAG as the buffer. Total assay volume was 400 μl. Separate aliquots of cell suspensions (4 × 10^6 cells/ml) and [^{3}H]SarCNU solution (hereafter called permeant) were preincubated for 15 min at 0°, 22°, and 37°. Transport was initiated by rapid addition of 200 μl of cell suspension to 200 μl of permeant (final SarCNU concentration, 50 μM) resting on 1.0 ml of Versilube F-50 silicone oil. Analysis of uptake, with time points greater than or equal to 20 sec, was performed with cell suspensions (2 × 10^6 cells/ml) that had been preincubated for 15 min at either 22° or 37° and to which an aliquot of [^{3}H]SarCNU had been added (final [^{3}H]SarCNU concentration, 50 μM). At indicated time points, assays were terminated by separating the cells from the medium by centrifugation in a Brinkman-Eppendorf microcentrifuge (14,000 × *g*) for 30 sec. A sample of medium was removed for liquid scintillation counting, followed by aspiration of medium and silicone oil with subsequent inversion of the microcentrifuge tubes. Contaminant medium was removed by wiping the inner wall twice with cotton-tipped applicators. Cell pellets were lysed with 100 μl of 4.0 M sodium acetate, pH 4.0, for 30 min. Cell debris was removed by centrifugation (14,000 × *g*) of the lysate for 3 min, and supernatant was placed in 10 ml of Ecolite(+) scintillation fluid (ICN) and counted in a Packard Tri-carb model 1500 liquid scintillation analyzer. The ICW was determined, after mixture of the cell suspension and unlabeled permeant with tritiated water and [*carboxyl*-^{14}C]inulin, by centrifugation of cells through silicone oil, as described previously (8, 13). Analysis of membrane-associated SarCNU radioactivity ("zero-time") was performed as described above, with immediate centrifugation after addition of the cell suspension to the [^{3}H]SarCNU suspension. Obtaining the zero-time point required 0.5 sec (as determined by metronome), and each time point was actually the time stated plus 0.5 sec (not including the time required for the cells to traverse the oil). [*carboxyl*-^{14}C]Inulin contamination, representing the extracellular water space within a cell pellet, contributed up to 0.33 ± 0.02 μl/1 μl of ICW at all temperatures, drug concentrations, and reagents tested. This medium contamination of cell pellets, determined for each individual experiment, was subtracted from every subsequent time point obtained. The accumulation of SarCNU in cells is expressed as the cell to medium ratio, which describes the distribution of SarCNU between 1 μl of the ICW and 1 μl of extracellular medium. Every time point, in each individual experiment, was measured in quadruplicate. Cell viability was determined by trypan blue exclusion to be >95% in all experiments.

Kinetic analysis and inhibition of uptake were performed at 37°, as described above, except that various concentrations of osmotically adjusted [^{3}H]SarCNU (final specific activity, 6.84–0.00684 μCi/μmol)

or osmotically adjusted agents tested for inhibition were used. The ICW was determined individually for any permeant containing concentrations greater than 1.0 mM, as described above. Analysis of transport was performed by rapid addition of 200 μl of cell suspension to 200 μl of permeant resting on silicone oil, with transport being terminated at the indicated time as described. Medium contamination of cell pellets was subtracted from every experimental determination, as described. The zero-time point was measured separately for every different drug concentration and inhibitor agent used. Initial transport velocity was measured at 2 sec (minus zero-time), during the linear phase of influx.

Analysis of the effects of metabolic poisons was carried out with 0.4 mM 2,4-dinitrophenol, 2.0 mM NaF, 2.0 mM iodoacetate, 0.4 mM ouabain, or 1.0 mM NaCN, as used previously (7, 14). Briefly, cells were pretreated for 15 min at 37° before examination of the uptake of [^{3}H] SarCNU, at a concentration of 50 μM, at zero-time and at 2 sec, as described. The ICW was determined individually for each different pretreatment, as described above.

Metabolism of [^{3}H]SarCNU. Samples obtained from transport assays were used in these experiments. Cell suspensions (2×10^6 cells/ml) were incubated with [^{3}H]SarCNU, at a 50 μM concentration (specific activity, 6.84 μCi/μmol), for 60 min at 37° and were separated from the permeant as described above. Cells were lysed in 15 μl of 4.0 M sodium acetate, pH 4.0, and stored immediately at −20° until needed. The cell pellets were analyzed for the presence of intact SarCNU by thin layer chromatography on no. 13179 silica gel plates (Kodak), using butan-1-ol/4.0 M sodium acetate, pH 4.0/water (4:2:1). In this system intact SarCNU has an R_F of 0.81.

Data analysis. Kinetic constants were determined by fitting the uncorrected influx data by nonlinear regression (15) with the following equation: $V = [(V_{max} \cdot S)/(S + K_m)] + (c \cdot S)$. The c term is the rate constant for nonfacilitated diffusion. Statistical analyses of influx rate, linear regression, and the two-tailed t test were performed with the CSS-Statistica computer program from StatSoft, Inc.

Results

Comparison of monolayer versus suspension techniques. Monolayer accumulation studies were initiated to study the uptake of [^{3}H]SarCNU at 37°, and uptake was found to be highly variable when normalized to cell number and protein content per plate. Pretreatment of culture plates with poly-D-lysine, fibronectin, and collagen was not able to increase the cell adherence to the substratum. However, the problem of cell adherence was not as apparent at 22°, which made it possible to compare the monolayer technique with the suspension technique that had been modified to perform accumulation studies at 37°. Fig. 1 shows that at 22°, in SK-MG-1 cells, both techniques proved to be reproducible, and it demonstrates that SarCNU accumulation was linear to 6 sec and began to plateau at 60 sec. Furthermore, the cell to medium ratio value was estimated for monolayers by harvesting cells with a rubber policeman and determining an ICW of 1.93 ± 0.21 μl/10^6 cells. This value obtained for monolayers is only an estimate, because cells on a monolayer are elongated, whereas cells in suspension are spherical. However, the estimated cell to medium ratio in monolayers, at 10 min, was similar to that determined in suspension (1.46 ± 0.08 versus 1.23 ± 0.06, respectively). After a 20-sec incubation with SK-MG-1 cells at 22°, drug added directly to cell suspension and the drug and cell suspension mixed over Versilube produced approximately equivalent cell to medium ratios of 0.97 ± 0.10 and 0.93 ± 0.10, respectively. The mixing of drug and cells over Versilube is the procedure that has been used for analysis of SarCNU uptake in SK-MG-1 cells, because with this method time points as early as 2 sec can be obtained, in contrast to 15 sec for direct addition of drug to the cell suspension.

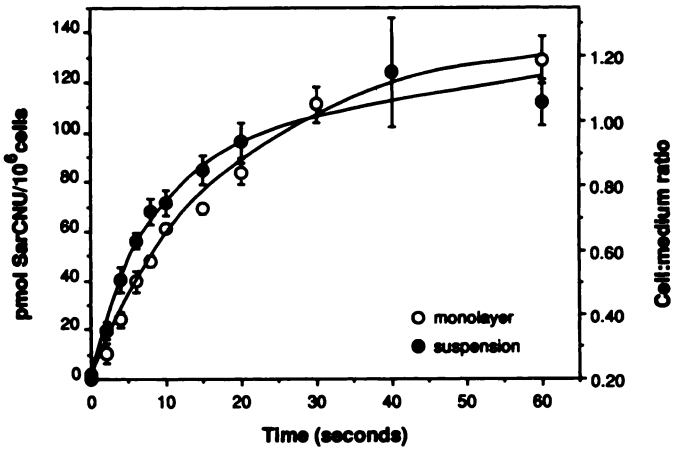

Fig. 1. Comparison of the time course of the uptake of [^{3}H]SarCNU, at a 50 μM concentration, by SK-MG-1 cells in suspension and in monolayers at 22°. The uptake in suspension is expressed as cell to medium distribution ratio, as described in Materials and Methods. The uptake in monolayers is expressed as pmol of SarCNU/10^6 cells. *Points*, mean of at least four separate experiments, with each time point assayed in quadruplicate; *bars*, standard error.

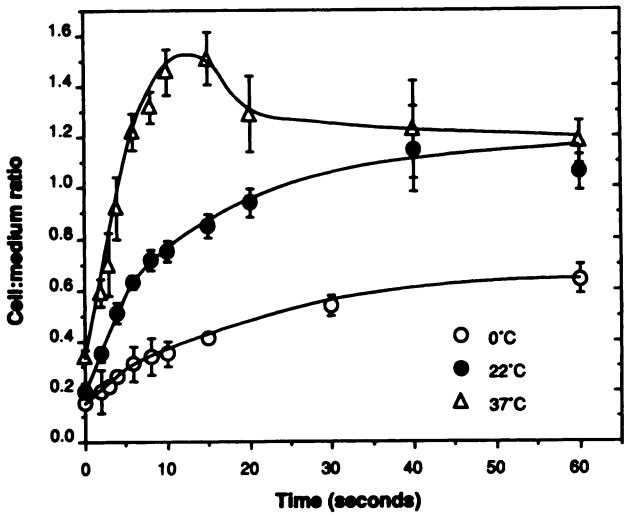

Fig. 2. Time course of uptake of [^{3}H]SarCNU, at a 50 μM concentration, by SK-MG-1 cells in suspension at 0°, 22°, and 37°. The uptake in suspension is expressed as cell to medium distribution ratio, as described in Materials and Methods. The zero-time cell to medium ratios were 0.150 ± 0.002, 0.20 ± 0.03, and 0.34 ± 0.02 for 0°, 22°, and 37°, respectively. *Points*, mean of at least four separate experiments, with each time point assayed in quadruplicate; *bars*, standard error.

Time course of uptake of [^{3}H]SarCNU at different temperatures. A time course of uptake of [^{3}H]SarCNU, at a 50 μM concentration, in SK-MG-1 cells in suspension at 0°, 22°, or 37° is shown in Fig. 2. At 37°, uptake of SarCNU was linear to 4 sec and began to reach equilibrium after 1 min. Additionally, the initial rate of accumulation at SarCNU concentrations as high as 50 mM was found to be linear to 4 sec (data not shown). The differences between the accumulation of SarCNU at 2 sec at various temperatures were found to be statistically significant. The cell to medium ratio values were 0.20 ± 0.08, 0.35 ± 0.03, and 0.59 ± 0.05 at 0°, 22°, and 37°, respectively ($p < 0.025$ for 37° versus 22° and 22° versus 0°; $p < 0.005$ for 37° versus 0°). All subsequent kinetic experiments

were carried out at 2 sec to approach the initial rate of uptake conditions and to minimize the effects of efflux, because the cell to medium ratio, minus the zero-time value, was below 0.3 at this point (16).

Steady state accumulation of [^{3}H]SarCNU. The cell to medium ratios in SK-MG-1 cells, at 22° and 37°, after a 30-min incubation with 50 μM [^{3}H]SarCNU were 1.08 ± 0.05 and 1.26 ± 0.07, respectively. These results, using a SarCNU concentration that approaches the theoretical PPC, are in agreement with previous accumulation studies that used 1 mM SarCNU and an insensitive colorimetric assay (8).

Chemical specificity of SarCNU transport. SarCNU has been demonstrated to competitively inhibit the uptake of sarcosinamide, which is transported into SK-MG-1 cells via the catecholamine uptake 2 carrier system (8). Consequently, epinephrine was tested for its ability to inhibit SarCNU accumulation under initial kinetic conditions. Additionally, several amino acids were tested as potential inhibitors of SarCNU uptake. A 500-fold molar excess concentration of lysine (Y^+ amino acid transport system), threonine (ASC and A amino acid transport systems), leucine (L-amino acid transport system), or glycine (glycine amino acid transport system) did not decrease the uptake of [^{3}H]SarCNU at a 50 μM concentration. A 10 mM concentration of SarCNU was able to inhibit the initial rate of influx of [^{3}H]SarCNU, at a 50 μM concentration, by 24%, whereas 10 mM epinephrine resulted in a 40% decrease in uptake of SarCNU (control velocity of 6.25 ± 0.50 versus 4.75 ± 0.25 pmol/μl of ICW/sec for 10 mM SarCNU, $p < 0.05$, and 3.75 ± 0.25 pmol/μl of ICW/sec for 10 mM epinephrine, $p < 0.005$). These results suggest that SarCNU and epinephrine are carried by a common transport system, as demonstrated previously (8).

Effect of sodium on uptake of [^{3}H]SarCNU in SK-MG-1 cells. The initial rate of accumulation at 37° of [^{3}H]SarCNU, at a 50 μM concentration, was analyzed for SK-MG-1 cells with either normal PAG containing 140 mM NaCl (151.2 meq/liter Na^+) or PAG containing 140 mM Tris, pH 7.4, replacing NaCl (11.2 meq/liter Na^+). In three paired experiments at 37°, the cell to medium ratio at 2 sec was 0.57 ± 0.05 in PAG with NaCl and 0.54 ± 0.05 in PAG with Tris. These findings indicate that the uptake of SarCNU is not dependent on the presence of sodium.

Effect of metabolic inhibitors on uptake of SarCNU. Various metabolic poisons were tested for their ability to inhibit the initial uptake of [^{3}H]SarCNU, at a 50 μM concentration. Pretreatment of SK-MG-1 cells with the metabolic poisons did not affect viability, with >95% of cells excluding trypan blue after a 60-min incubation at 37°. In four paired experiments, pretreatment of cells with 2,4-dinitrophenol, NaF, iodoacetate, ouabain, or NaCN did not affect the initial uptake of SarCNU (119 ± 18, 123 ± 11, 118 ± 9, 96 ± 11, and 112 ± 10% of control uptake, respectively).

Kinetic analysis of [^{3}H]SarCNU uptake. As the SarCNU concentration was increased from 50 μM to 7.5 mM the cell to medium ratio, at 2 sec, decreased from 0.63 to 0.52. Thereafter, there was no additional decrease in SarCNU distribution up to a 50 mM concentration (data not shown). The inability to reduce the cell to medium ratio of SarCNU below approximately 0.52 at concentrations of >7.5 mM is consistent with a large nonfacilitated diffusion rate of SarCNU in SK-MG-1 cells. Therefore, SarCNU influx can be seen to result from two processes, one exhibiting saturability at low concentrations and one exhibiting nonsaturability at high concentrations. The relationship between initial rate of SarCNU influx and SarCNU concentration is shown in Fig. 3. The nonfacilitated influx rate of SarCNU at high concentrations could not be determined accurately, because at concentrations of >10 mM SarCNU the ICW volume was affected. However, when high concentrations of epinephrine (10 mM), which did not affect ICW, were present in the assay mixture, the residual influx rates of SarCNU were observed to be linearly dependent on SarCNU concentration (Fig. 3). These residual influx rates were interpreted to represent the nonfacilitated diffusion of SarCNU, giving the derived nonfacilitated influx rate of 61.98 ± 2.09 pmol/μl of ICW/sec/mM SarCNU. This rate constant was then used to correct experimental values for nonfacilitated diffusion (Fig. 3). The corrected rate of SarCNU influx into SK-MG-1 cells follows Michaelis-Menten kinetics, and nonlinear regression analysis of the uncorrected data estimated a K_m of 2.39 ± 0.37 mM and a V_{max} of 236 ± 53 pmol/μl of ICW/sec.

Dixon plot analysis of SarCNU and epinephrine transport. The Dixon plot analysis of the effect of increased concentrations of unlabeled external epinephrine on the uptake [^{3}H]SarCNU, at concentrations of 50 and 200 μM, by SK-MG-1 cells at 37° is shown in Fig. 4. The influx of SarCNU was corrected for nonfacilitated diffusion in all cases. The point of intersection of the two plots is consistent with competitive inhibition of SarCNU uptake by epinephrine and gives a K_i of 214 μM when all experimental values are averaged. The mean K_i determined from five independent experiments was found to be 163 ± 15 μM. Furthermore, the K_i is similar to the K_m of epinephrine in SK-MG-1 cells, which was determined to be 270 μM without correction for diffusion (8).

Metabolism of SarCNU. Thin layer chromatography was used to assess breakdown of 50 μM [^{3}H]SarCNU in the medium and intracellularly after a 60-min incubation, at 37°, of SarCNU

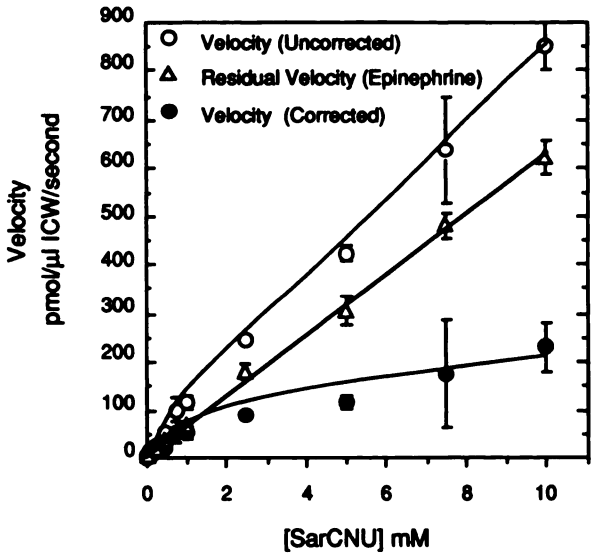

Fig. 3. Uptake of [^{3}H]SarCNU as a function of concentration. The 2-sec uptake of SarCNU, at concentrations from 50 μM to 10 mM, in the presence (residual velocity) and absence (velocity) of 10 mM epinephrine was measured in suspension at 37°, as described in Materials and Methods. The corrected curve represents the velocity for 50 μM to 10 mM SarCNU minus the residual velocity for SarCNU in the presence of epinephrine. *Points*, mean of at least six separate experiments, with each drug concentration assayed in quadruplicate; *bars*, standard error.

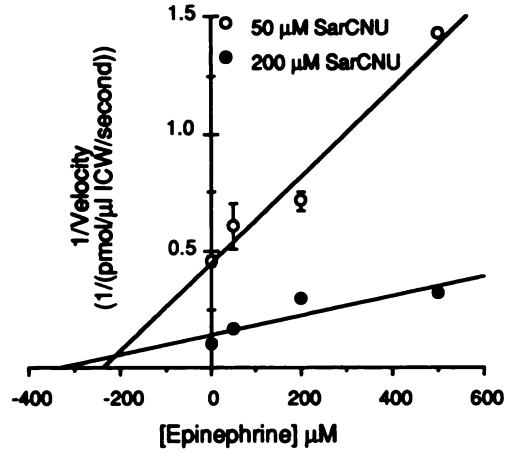

Fig. 4. Dixon plot of epinephrine inhibition of [^{3}H]SarCNU. The 2-sec uptake of [^{3}H]SarCNU, at concentrations of 50 and 200 μM, in medium containing 50–500 μM epinephrine was measured in suspension at 37°, as described in Materials and Methods. *Points*, mean of six separate experiments, with each drug concentration assayed in quadruplicate; *bars*, standard error.

with SK-MG-1 cells. Before incubation with cell suspensions, SarCNU was 99% pure, with an R_F of approximately 0.81. After a 60-min incubation, the SarCNU present in the medium was 99% pure and migrated with a similar R_F, compared with intact drug. This observation is consistent with the half-life of SarCNU in pH 7.4 medium, which has been shown to be approximately 5.5 hr (1). Analysis of cellular lysates in four independent experiments revealed that SarCNU had been metabolized to a limited extent. The percentage of intact SarCNU in cell lysates after a 60-min incubation was 81.5 ± 4.59%. These results indicate that there is no significant metabolism of SarCNU during the 2-sec incubation necessary to measure the initial velocity of SarCNU influx.

Discussion

The objective of the present study was to define the transport of an experimental antitumor compound, SarCNU, in a human glioma cell line, SK-MG-1, that is sensitive to SarCNU. Previously, it was suggested that SarCNU may be transported into human glioma cells by a process that mediates the uptake of catecholamines and sarcosinamide, the carrier moiety present in SarCNU (8). These studies provided indirect evidence suggesting that SarCNU enters cells via carrier-mediated uptake, compared with BCNU and other CENUs, which enter cells via passive diffusion (5). Radiolabeled SarCNU was used to directly assess the characteristics of uptake of SarCNU in suspension, using a modification of oil-stop methodology that is highly reproducible.

The uptake of SarCNU in SK-MG-1 cells was found to be temperature dependent, sodium independent, and inhibited by excess SarCNU. Furthermore, the accumulation does not proceed against a concentration gradient (cell to medium ratio slightly above unity), suggesting that SarCNU enters cells via a nonconcentrative mechanism. Additionally, metabolic poisons were unable to inhibit the influx of SarCNU, indicating that accumulation of SarCNU is not dependent on an energy source.

The chemical specificity of SarCNU accumulation was investigated by taking into consideration the structure of this experimental drug, which contains an amino acid amide, sarcosinamide. None of the amino acids tested were able to inhibit SarCNU uptake, indicating that SarCNU does not enter SK-MG-1 cells by these carrier systems. Previous results demonstrated that SarCNU was able to competitively inhibit the accumulation of sarcosinamide (K_i = 3.26 mM), which in turn shares a common carrier with epinephrine (8). This system has been shown to mediate the facilitated diffusion of epinephrine and sarcosinamide by a saturable and sodium-independent carrier (8). Therefore, the influence of excess concentrations of epinephrine on SarCNU influx was investigated. Epinephrine was able to inhibit the accumulation of SarCNU, suggesting that SarCNU, sarcosinamide, and epinephrine share a common carrier system in SK-MG-1 cells. These results are in agreement with our previous observation that the cytotoxicity of SarCNU in SK-MG-1 cells is not decreased in the presence of excess amino acids but is decreased in the presence of sarcosinamide (7).

Analysis of the velocity of uptake versus the substrate concentration curve of SarCNU suggests that accumulation of SarCNU is a combination of saturable (facilitated) and nonsaturable (nonfacilitated) uptake systems. The influence of high concentrations of SarCNU on the estimation of ICW prevented direct measurement of the rate coefficient of nonfacilitated diffusion. However, because epinephrine is able to inhibit the accumulation of SarCNU to a greater extent than is SarCNU itself, epinephrine was used to estimate the nonfacilitated diffusion rate for SarCNU. Epinephrine was chosen because it was thought to be the physiological substrate of the carrier system. In the presence of excess epinephrine, the accumulation of SarCNU was linearly dependent on concentration and yielded an estimate of the nonfacilitated diffusion rate of SarCNU. At the theoretical PPC of SarCNU, nonfacilitated diffusion is estimated to contribute approximately 50% of the total influx rate.

Epinephrine was found to inhibit the transport of SarCNU in a competitive fashion, with a K_i similar to the K_m value determined in SK-MG-1 cells (8). These results are consistent with there being a common transporter for epinephrine, SarCNU, and sarcosinamide. Previously, Skalski *et al.* (8) suggested that epinephrine and sarcosinamide share a common catecholamine carrier. Furthermore, it was pointed out that catecholamine transport similar to that described for sarcosinamide has been described in a number of tissues in the central and peripheral nervous system. The transport is saturable and sodium independent, and the K_m values for epinephrine and norepinephrine range from 2 to 250 μM, depending on the type of tissue studied. Substitution of the hydrogens of the catecholamine amino group with hydrocarbons, as with the *N*-methyl group of epinephrine and sarcosinamide, greatly enhances the uptake by this transport system (17–19). The *N*-methyl group present in sarcosinamide, the carrier moiety of SarCNU, presumably allows SarCNU to be recognized and subsequently transported by the catecholamine carrier present in SK-MG-1 cells.

The results of this study provide evidence that SarCNU uptake is mediated by an epinephrine-sensitive carrier that functions, physiologically, to transport catecholamines. This is the first direct demonstration of carrier-mediated uptake of a CENU. However, other transport systems have been observed for alkylating anticancer compounds such as Melphalan (20),

nitrogen mustard (21), and cyclophosphamide (14). The transport of SarCNU is similar to that characterized for cyclophosphamide; both compounds appear to enter cells by facilitated diffusion at low concentrations and by a technically nonsaturable (nonfacilitated) mechanism at high doses (14). The presence of the SarCNU carrier in SK-MG-1 cells may be responsible for the 2-fold higher steady state concentration of SarCNU versus BCNU (8), which enters cells via passive diffusion (5). Because the catecholamine transporter is found predominantly in tissues of the nervous system (17–19) and is clearly absent in a human lung cancer cell line and human fibroblasts (22), this may account for the increased cytotoxicity of SarCNU in gliomas.

References

1. Suami, T., T. Kato, and T. Hisamatsu. 2-Chloroethylnitrosourea congeners of amino acid amides. *J. Med. Chem.* **25**:829–832 (1982).
2. Skalski, V., J. Rivas, L. C. Panasci, and W. Feindel. The cytotoxicity of sarcosinamide chloroethylnitrosourea (SarCNU) and BCNU in primary gliomas and glioma cell lines: analysis of data in reference to theoretical peak plasma concentrations in man. *Cancer Chemother. Pharmacol.* **22**:137–140 (1988).
3. Houchens, D., M. Sheridan, R. Nines, M. Finfrock, and N. Trigg. Glioma and medulloblastoma xenografts as models for brain tumor drug development, in *The Sixth International Workshop on Immunodeficient Animals*. Karger Publishing, Basel, Switzerland, 157–161 (1989).
4. Panasci, L. C., M. Dufour, L. Chevalier, G. Isabel, P. Lazarus, A. McQuillan, E. Arbit, S. Brem, and W. Feindel. Utilization of the HTSCA and CFU-C assay to identify two new 2-chloroethylnitrosourea congeners of amino acid amides with increased *in vitro* activity against human gliomas compared with BCNU. *Cancer Chemother. Pharmacol.* **14**:156–159 (1985).
5. Begleiter, A., H.-Y. P. Lam, and G. J. Goldenberg. Mechanism of uptake of nitrosourea by L5178Y lymphoblasts *in vitro*. *Cancer Res.* **37**:1022–1027 (1977).
6. Vistica, D. T. Cytotoxicity as an indicator for transport mechanism: evidence that Melphalan is transported by two leucine-preferring carrier systems in the L1210 murine leukemia cell. *Biochim. Biophys. Acta* **550**:309–317 (1979).
7. Skalski, V., W. Feindel, and L. C. Panasci. The cytotoxicity of a 2-chloroethylnitrosourea analog of sarcosinamide in the SK-MG-1 human glioma cell line as a possible indicator for transport. *J. Neuro-Oncol.* **7**:189–193 (1989).
8. Skalski, V., W. Feindel, and L. C. Panasci. Transport of amino acid amide sarcosinamide and sarcosinamide chloroethylnitrosourea in human glioma SK-MG-1 cells. *Cancer Res.* **50**:3062–3066 (1990).
9. Domin, B. A., W. B. Mahony, and T. P. Zimmerman. Purine nucleobase transport in human erythrocytes: reinvestigation with a novel "inhibitor-stop" assay. *J. Biol. Chem.* **263**:9276–9284 (1988).
10. Dixon, M. The determination of enzyme inhibitor constants. *Biochem. J.* **55**:170–171 (1953).
11. Ronquist, G., G. Argen, J. Ponten, and B. Westermark. α- Aminoisobutyric acid transport into human glia and glioma cells in culture. *J. Cell. Physiol.* **89**:433–440 (1976).
12. Paterson, A. R. P., N. Kolassa, and C. E. Cass. Transport of nucleoside drugs in animal cells. *Pharmacol. Ther.* **12**:515–536 (1981).
13. Wolhueter, R. M., R. Marz, J. C. Graff, and P. G. Plagemann. The application of rapid kinetic techniques to the transport of thymidine and 3-*O*-methylglucose into mammalian cells in suspension culture. *J. Cell. Physiol.* **89**:605–612 (1976).
14. Goldenberg, G. J., H. B. Land, and D. V. Cormack. Mechanism of cyclophosphamide transport by L5178Y lymphoblasts *in vitro*. *Cancer Res.* **34**:3274–3282 (1974).
15. Leatherbarrow, R. J. Use of nonlinear regression to analyze enzyme kinetic data: application to situations of substrate contamination and background subtraction. *Anal. Biochem.* **184**:274–278 (1990).
16. Stein, W. D. Facilitated diffusion: the simple carrier, in *Transport and Diffusion across Membranes*. Academic Press, Inc., Montreal, 267 (1986).
17. Iverson, L. L. Catecholamine uptake processes. *Br. Med. Bull.* **29**:130–135 (1973).
18. Pelton, E. W., H. K. Kimelberg, S. V. Shipherd, and R. S. Bourke. Dopamine and norepinephrine uptake and metabolism in astroglial cells in culture. *Life Sci.* **28**:1655–1663 (1981).
19. Kimelberg, H. K. Occurence and functional significance of serotonin and catecholamine uptake in astrocytes. *Biochem. Pharmacol.* **35**:2273–2281 (1986).
20. Redwood, W. R., and M. Colvin. Transport of Melphalan by sensitive and resistant L1210 cells. *Cancer Res.* **40**:1144–1149 (1980).
21. Goldenberg, G. J., C. L. Vanstone, L. G. Israels, D. Ilse, and I. Bihler. Evidence for a transport carrier of nitrogen mustard in nitrogen mustard-sensitive and -resistant L5178Y lymphoblasts. *Cancer Res.* **30**:2285–2291 (1970).
22. Malapetsa, A., J. L. Bramson, A. J. Noë, and L. C. Panasci. Lack of evidence for a high-affinity sarcosinamide carrier or a catecholamine carrier in Calu-1 lung-cancer cells, HT-29 colon-cancer cells, and DHF fibroblasts. *Cancer Chemother. Pharmacol.* **31**:146–150 (1992).

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