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# Characterization of the Catecholamine Extraneuronal Uptake$_2$ Carrier in Human Glioma Cell Lines SK-MG-1 and SKI-1 in Relation to (2-Chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) Selective Cytotoxicity

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**ABSTRACT.** Transport of (2-chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) and (−)-norepinephrine was investigated in SarCNU-sensitive SK-MG-1 and -resistant SKI-1 human glioma cell lines. [$^3$H]SarCNU influx was inhibited by SarCNU, sarcosinamide, and (±)-epinephrine in SK-MG-1 cells with competitive inhibition observed by (±)-epinephrine ($K_i$ = 140 ± 12 μM) and (±)-norepinephrine ($K_i$ = 255 ± 41 μM). No effect on influx was detected in SKI-1 cells. [$^3$H](−)-Norepinephrine influx was linear to 15 sec in both cell lines and temperature dependent only in SK-MG-1 cells. Influx of [$^3$H](−)-norepinephrine was found to be saturable in SK-MG-1 ($K_m$ = 148 ± 28 μM, $V_{max}$ = 1.23 ± 0.18 pmol/μL intracellular water/sec) but not in SKI-1 cells. In SK-MG-1 cells, [$^3$H](−)-norepinephrine influx was found to be inhibited competitively by (−)-epinephrine ($K_i$ = 111 ± 7 μM) and SarCNU ($K_i$ = 1.48 ± 0.22 mM). Ouabain and KCl were able to inhibit the [$^3$H](−)-norepinephrine influx in SK-MG-1 cells, consistent with influx being driven by membrane potential. Several catecholamine uptake$_2$ inhibitors were able to reduce significantly the influx of [$^3$H](−)-norepinephrine and [$^3$H]SarCNU with no inhibition by a catecholamine uptake$_1$ inhibitor. These findings suggest that increased sensitivity of SK-MG-1 to SarCNU is secondary to enhanced accumulation of SarCNU mediated via the catecholamine extraneuronal uptake$_2$ transporter, which is not detectable in SKI-1 cells. The introduction of SarCNU into clinical trials will confirm if increased uptake via the catecholamine extraneuronal uptake$_2$ transporter will result in increased antitumor activity. BIOCHEM PHARMACOL 51;12:1639–1648, 1996.

**KEY WORDS.** chloroethylnitrosourea; glioma; uptake$_2$; norepinephrine; epinephrine

More than half of the patients that develop malignant brain tumors present with malignant high-grade astrocytomas [1]. The current standard therapy for patients with high-grade astrocytomas involves surgical resection followed by radiotherapy. The addition of chemotherapy to this therapy has improved only marginally the 1-year survival rate of patients. It has been determined recently that the addition of chemotherapy to surgery and radiation improved the median survival duration of patients from 9.4 to 12 months [2]. The CENUs† are the most active single agents for treatment of malignant astrocytomas. These drugs are relatively lipid-soluble, non-ionized, and readily cross the blood–brain barrier [1]. BCNU is currently one of the most active CENUs [1, 3–5].

The CENUs result in dose-limiting toxicity of delayed cumulative myelosuppression [6]. The development of CENUs that are more active against gliomas and less myelotoxic would be very useful. SarCNU is a novel derivative of CENU that contains the amino acid amide group, *N*-methylglycinamide, known as sarcosinamide [7]. SarCNU has been shown previously to be more active than BCNU in primary gliomas plus glioma cell lines *in vitro* [8] and in a human glioma cell line intracerebrally implanted into nude mice [9]. Moreover, SarCNU is less toxic than BCNU in mice [7] and less myelotoxic in the *in vitro* colony forming unit (cell) assay with normal human bone marrow [10]. Furthermore, SarCNU is more toxic *in vitro*, to primary

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† *Abbreviations:* BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CENU, chloroethylnitrosourea; COMT, catechol-O-methyltransferase; DPBS, Dulbecco's incomplete phosphate-buffered saline; ICW, intracellular water; MAO, monoamine oxidase; PAG, Dulbecco's incomplete phosphate-buffered saline supplemented with 0.7% BSA fraction V, 0.25% dextrose and 0.001% phenol red, pH 7.4; PPC, peak plasma concentration; and SarCNU, (2-chloroethyl)-3-sarcosinamide-1-nitrosourea.

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human gliomas, at its theoretical PPC of 68 μM compared with BCNU at its clinically achievable PPC of 9 μM [8].

The clinically available CENUs, which include BCNU, CCNU, and methyl-CCNU, all enter cells via passive diffusion [11]. The presence of the sarcosinamide carrier moiety in SarCNU suggests that it may enter cells through a mechanism other than purely passive diffusion. This has been supported by the fact that the presence of excess sarcosinamide during *in vitro* cytotoxicity assays increases the 70% inhibitory concentration of SarCNU but not that of BCNU [12]. Initial investigations with [$^3$H]sarcosinamide suggested that sarcosinamide enters cells through an epinephrine-sensitive carrier system and that SarCNU was able to inhibit this uptake competitively [13].

The transport of [$^3$H]SarCNU has been assessed in two human glioma cell lines, SK-MG-1 and SKI-1. The analysis of the sensitivity of these cell lines to SarCNU and BCNU revealed that SK-MG-1 cells are more sensitive than SKI-1 cells [8, 14]. Influx of [$^3$H]SarCNU into SK-MG-1 cells has been shown to be saturable, temperature dependent, sodium independent, and competitively inhibited by (±)epinephrine [15]. However, influx of [$^3$H]SarCNU into SKI-1 cells was found to occur through passive diffusion [14]. The steady-state accumulation of SarCNU, at 37°, was found to be increased significantly by 54% in SK-MG-1 compared with SKI-1 cells. This difference in accumulation, at 37°, was found to be associated with increased influx of SarCNU into SK-MG-1 versus SKI-1 cells since the rate of efflux of [$^3$H]SarCNU from both cell lines was found not to be significantly different. In addition, there was minimal metabolism in either cell line after a 60-min incubation. Examination of the cytotoxicity of SarCNU demonstrated that the increased sensitivity of SK-MG-1 versus SKI-1 cells was due to the presence of a SarCNU carrier identifiable in SK-MG-1 but not in SKI-1 cells [14]. The SarCNU carrier may be the extraneuronal catecholamine uptake$_2$ transporter.

Many synapses in the CNS are surrounded by the processes of astroglial cells (astrocytes) [16]. The perisynaptic location of astrocytes is thought to indicate that they may play a role in transmitter reuptake at the neuronal junction [17–20]. Since uptake into extraneuronal sites is followed by metabolism by MAO and COMT, the role that astroglial cells play in the reuptake of catecholamines has not been well defined due to insufficient experimental data [16, 21]. However, a low-affinity, temperature-sensitive, sodium-independent, and non-concentrative (non-active) mode of norepinephrine uptake has been demonstrated in rat neonatal primary astrocytes [22]. Additionally, saturable transport of a potent but non-selective uptake$_2$ substrate has been identified in various primary human gliomas, but this transport has not been characterized extensively [20, 23].

In this investigation, [$^3$H]SarCNU and [$^3$H](−)norepinephrine influx and metabolism have been characterized to determine if the altered influx of SarCNU is due to altered expression of a specific catecholamine transporter in SK-MG-1 and SKI-1 human glioma cell lines. The specificity and kinetics of [$^3$H]SarCNU and [$^3$H](−)norepinephrine transport were examined in SK-MG-1 and SKI-1 cells in order to determine the identity of the catecholamine carrier and its role in the selective cytotoxicity of SarCNU.

## MATERIALS AND METHODS

### *Drugs*

SarCNU (NSC 364432) was a gift from Dr. T. Suami, Keio University, Japan [7]. It was dissolved in 0.001 M citrate buffer, pH 4.0, divided into aliquots, and stored at −20°.

### *Materials*

(±)-Epinephrine HCl, (±)-norepinephrine HCl, (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, sarcosinamide HCl, papaverine HCl, cimetidine HCl, and desipramine HCl were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Normetanephrine (NMN), 3,4-dihydroxyphenylglycol (DOPEG), 3-methoxy-4-hydroxyphenylglycol (MOPEG), 3-methoxy-4-hydroxymandelic acid (VMA), 3,4-dihydroxymandelic acid (DOMA), and ouabain octahydrate were purchased from Anachemia (Montréal, Québec, Canada). (2-Chloroethyl)-3-[$^3$H]sarcosinamide-1-nitrosourea ([$^3$H]SarCNU, 342 mCi/mmol) was prepared by Amersham Laboratories (Buckinghamshire, England), using the technique described by Suami *et al.* [7]. The radiochemical purity was greater than 99%, as determined 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) with an $R_f$ value of 0.81. [7-$^3$H](−)-Norepinephrine (11.9 Ci/mmol) was purchased from Dupont NEN (Mississauga, Ontario, Canada). The radiochemical purity was greater than 95%, as determined by the cascade column chromatographic technique of Graefe *et al.* [24]. [*carboxyl*-$^{14}$C]Inulin (3.2 mCi/mmol), tritiated water (1 Ci/mL), sulforhodamine B, and bovine serum albumin (Fraction V, powder; low salt and salt-free fractions) were purchased from ICN (Costa Mesa, CA, U.S.A.). McCoy's 5A modified medium, fetal bovine serum, and DPBS were supplied by Canadian Life Technologies (GIBCO, Montréal, Québec, Canada). Versilube F-50 silicone oil was from Nessa Products (Montréal, Québec, Canda). Dextrose, sodium acetate, HCl, KCl, and NaCl were from Fisher Laboratories (Ottawa, Ontario, Canada). Alumina and Dowex 50Wx4 were purchased from Aldrich (Milwaukee, WI, U.S.A.). Disprocynium-24 was a gift from Dr. E. Schömig (University of Würzburg, Würzburg, Germany).

### *Cell Culture*

SK-MG-1 and SKI-1 human glioma cell lines were grown as described [14]. 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 × $10^6$ cells/mL, as needed.

### *Metabolism of [7-$^3$H](−) Norepinephrine*

The procedure for preparing medium and cell lysate involved adding 1.0 mL of [$^3$H](−)-norepinephrine, at a 0.1 μM, 50 μM, or 20 mM concentration (sp. act. 11.9 Ci/mmol, 23.8 mCi/mmol, or 59.5 μCi/mmol, respectively), to 1.0 mL of cell suspension (4 × $10^6$ cells/mL). The suspensions were incubated for 5, 10, and 15 sec at 37°. At the appropriate time, 10 mL of ice-cold PBS was added, and the suspensions were centrifuged at 30 *g* for 2 min at 4°. Immediate additions of ice-cold PBS was utilized for the "zero-time" metabolism point. Medium samples were collected from the supernatant to which HCl and ethanol were added to a final concentration of 0.2 M HCl:ethanol (9:1). After the remaining supernatant was aspirated, cell pellets were washed with 1 mL of ice-cold PBS and immediately centrifuged at 300 *g* for 2 min at 4°. Following aspiration, the cell pellets were lysed with 100 μL of 0.2 M HCl:ethanol (9:1). All samples were stored at −20°, and chromatographic analysis was done within 24 hr. Prior to chromatography, 900 μl of 2 M sodium acetate, pH 8.2, was mixed with the lysate, and (−)-norepinephrine, NMN, DOPEG, MOPEG, VMA, and DOMA were added to a final concentration of 50 μM to act as carriers. The samples were centrifuged at 14,000 rpm in a Brinkmann Eppendorf microcentrifuge for 3 min at room temperature. The supernatant was then applied to the cascade chromatography technique. The contents of [$^3$H]norepinephrine and its metabolites in medium and cell lysates were determined with the cascade column chromatography technique [24, 25].

### *Norepinephrine Transport Experiments*

A modified version of "oil-stop" methodology was used to assay transport of [$^3$H](−)-norepinephrine at a concentration of 25 μM (sp. act. 23.8 mCi/mmol) in the glioma cells, in suspension, at 0 and 37°, as described previously [15, 26]. The accumulation of norepinephrine in cells is expressed as the cell to medium ratio which is the concentration of norepinephrine in 1 μL of the ICW as compared to 1 μL of the extracellular medium. The ICW was determined by mixing the cell suspension and unlabeled permeant with tritiated water and [*carboxyl*-$^{14}$C]inulin, followed by centrifugation of cells through silicone oil, as described previously [14, 15]. [*carboxyl*-$^{14}$C]Inulin contamination, representing the extracellular water space within a cell pellet, contributed up to 0.33 ± 0.02 and 0.32 ± 0.02 μL/1 μL of ICW at all temperatures and drug concentrations used for SK-MG-1 and SKI-1 cells, respectively. This medium contamination of cell pellets, determined for each individual experiment, was subtracted from every subsequent time point obtained. Every time point, in each individual experiment, was performed in quadruplicate. Cell viability was determined, by trypan blue exclusion, to be greater than 95% in all experiments.

Kinetic analysis and inhibition of norepinephrine influx were performed as described above, except that various concentrations of osmotically adjusted norepinephrine (final sp. act. 11.9 mCi/mmol to 29.8 μCi/mmol) or osmotically adjusted agents tested for inhibition were used in the assay. The ICW was determined, individually, for any permeant containing concentrations greater than 1.0 mM, as described above. Medium contamination of cell pellets were subtracted from every experimental determination, as described. The zero-time point was measured separately for every different drug concentration used. Initial transport velocity was measured at 5 sec (minus zero-time), during the linear phase of influx.

Analysis of the effect of the metabolic poison ouabain (0.4 mM) was carried out as previously described [15]. Analysis of the effect of 100 mM KCl replacing NaCl in isoosmotically adjusted PAG was carried out as previously utilized [27]. Briefly, cells were pretreated for 15 min at 37°, prior to examining the uptake of [$^3$H]norepinephrine, at a concentration of 25 μM, at "zero-time" and 5 sec, as described [14, 15]. The ICW was determined, individually, for each pretreatment, as above.

### *SarCNU Transport Experiments*

Kinetic analysis and inhibition of influx, at 2 sec, of [$^3$H]SarCNU, at concentrations of 50 and 200 μM (final sp. act. 6.84 and 1.71 μCi/μmol, respectively), was performed at 37°, as described above, except that various osmotically adjusted agents tested for inhibition were used.

Analysis of the effect of 100 mM KCl replacing NaCl in isoosmotically adjusted PAG on [$^3$H]SarCNU influx, at a 50 μM concentration (sp. act. 6.84 μCi/μmol), was performed as described above.

### *Calculations*

Kinetic constants were determined by fitting the uncorrected influx data by nonlinear regression 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. The nonlinear regression was performed utilizing Kaleida Graph (Abelbeck Software). Statistical analysis of influx rate, linear regression, ANOVA analysis, and paired and unpaired two-tailed *t*-tests were performed utilizing StatView 512+ (BrainPower, Inc., Calabasas, CA, U.S.A.).

## RESULTS

### *Inhibition of [$^3$H]SarCNU Accumulation*

It has been observed that the presence of excess sarcosinamide during 60-min SarCNU cytotoxicity assays reduces the effectiveness of SarCNU in SK-MG-1 cells [12]. Therefore, the influx of [$^3$H]SarCNU, at a concentration of 50 μM, over time in the presence and absence of 10 mM sarcosinamide was analyzed (Fig. 1). The area under the curve of total intracellular SarCNU exposure over time was reduced significantly by 9% ($P = 0.0362$, 86 and 78 cell to medium ratio units for influx in the absence and presence of

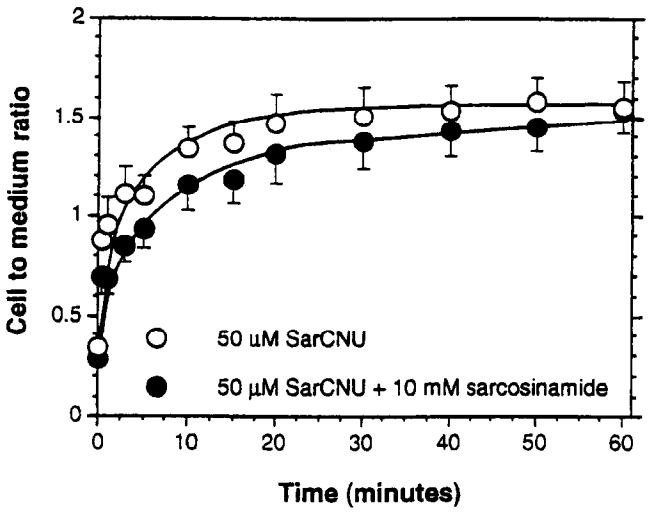


**FIG. 1. Comparison of the 60-min time–course of the influx of [$^3$H]SarCNU, at a 50 µM concentration, by SK-MG-1 cells in the presence and absence of 10 mM sarcosinamide at 37°. The uptake, in suspension, is expressed as cell:medium distribution ratio, as described in Materials and Methods. Points: means of at least six separate experiments with each time interval performed in quadruplicate; bars: SE.**

sarcosinamide, respectively). Additionally, analysis of the inhibition of the initial rate of [$^3$H]SarCNU influx, at a 50 µM concentration, into SK-MG-1 and SKI-1 cells is presented in Table 1. There was 30–40% inhibition of influx of [$^3$H]SarCNU into SK-MG-1 cells in the presence of SarCNU, sarcosinamide, or epinephrine ($P < 0.005$). However, the influx of [$^3$H]SarCNU into SKI-1 cells was not inhibited significantly by any of the compounds.

### *Dixon Plot Analysis of SarCNU Transport*

In SK-MG-1 cells, the Dixon plot analysis [28] of the effect of increasing concentration of (±)-epinephrine and (±)-norepinephrine on the uptake of [$^3$H]SarCNU, at concentrations of 50 and 200 µM, at 37° is shown in Fig. 2. The influx of SarCNU was corrected for nonfacilitated diffusion in all cases [15]. The point of intersection of the two regression lines for (±)-epinephrine and (±)-norepinephrine was consistent with competitive inhibition of SarCNU influx by both compounds. The mean $K_i$ for three independent experiments was found to be 140 ± 12 and 255 ± 41 µM for (±)-epinephrine and (±)-norepinephrine, respectively. The $K_i$ value determined for (±)-epinephrine was similar to the $K_i$ value determined previously [15].

### *Metabolism of [$^3$H](−)-Norepinephrine*

The intracellular metabolism of [$^3$H](−)-norepinephrine has to be determined since COMT and MAO are involved in the normal breakdown of norepinephrine [21]. Therefore, cascade column chromatography was used to assess the breakdown of [$^3$H](−)-norepinephrine in the medium and, intracellularly, in both SK-MG-1 and SKI-1 cells. Preliminary metabolism studies with 0.1 µM [$^3$H](−)norepinephrine were performed at 37°. The addition of up to 40 µM U-0521, a COMT inhibitor, for a 30-min metabolism study demonstrated a recovery of 51% intact norepinephrine in SK-MG-1 versus 20% in untreated cells and 92% in all media. The addition of up to 40 µM pargyline, an MAO inhibitor, did not increase the recovery of intact norepinephrine in SK-MG-1 cells. Furthermore, inhibition with 10 µM U-0521 plus 10 µM pargyline did not increase significantly the recovery of intact norepinephrine versus 10 µM U-0521 in SK-MG-1 cells (approximately 42%). In SKI-1 cells, recovery of intact [$^3$H](−)-norepinephrine increased to 77% in the presence of 10 µM U-0521 or 10 µM U-0521 plus 10 µM pargyline (control was 40%).

Since the COMT inhibitor U-0521 has been shown to be metabolized by COMT and to interfere with initial rates of influx of norepinephrine [29, 30], the time–course of metabolism of [$^3$H](−)-norepinephrine, at 25 µM and 10 mM concentrations without inhibitors, was performed at 37°. In the medium, both drug concentrations had 84 ± 2% average intact [$^3$H](−)-norepinephrine following the appropriate incubations. The recovery of intact intracellular

**TABLE 1. Chemical specificity of the influx of SarCNU, at concentration of 50 µM, in SK-MG-1 and SKI-1 cells**

| Cell line | Compound (10 mM) | N* | Initial velocity† (pmol/µL ICW/sec) | % Control‡ | P§ |
|---|---|---|---|---|---|
| SK-MG-1 | | 8 | 3.95 ± 0.35 | | |
| | SarCNU | 8 | 2.90 ± 0.45 | 70 ± 7 | <0.005 |
| | Sarcosinamide | 8 | 2.25 ± 0.35 | 56 ± 6 | <0.005 |
| | Epinephrine | 8 | 2.55 ± 0.35 | 63 ± 6 | <0.005 |
| SKI-1 | | 8 | 2.98 ± 0.20 | | |
| | SarCNU | 8 | 3.0 ± 0.30 | 103 ± 9 | NS‖ |
| | Sarcosinamide | 8 | 2.85 ± 0.30 | 98 ± 10 | NS |
| | Epinephrine | 8 | 2.6 ± 0.3 | 88 ± 10 | NS |

SK-MG-1 and SKI-1 cells in suspension were examined at 37° at 2 sec for uptake of 50 µM [$^3$H]SarCNU in the absence or presence of the indicated inhibitors, as described in Materials and Methods.

* Number of individual experiments.

† Velocity values (mean ± SEM) were not corrected for diffusion.

‡ Percent difference (mean ± SEM) between control velocity versus velocity in the presence of the compound tested for inhibition.

§ Significance as determined by the Scheffé test.

‖ Not significant.

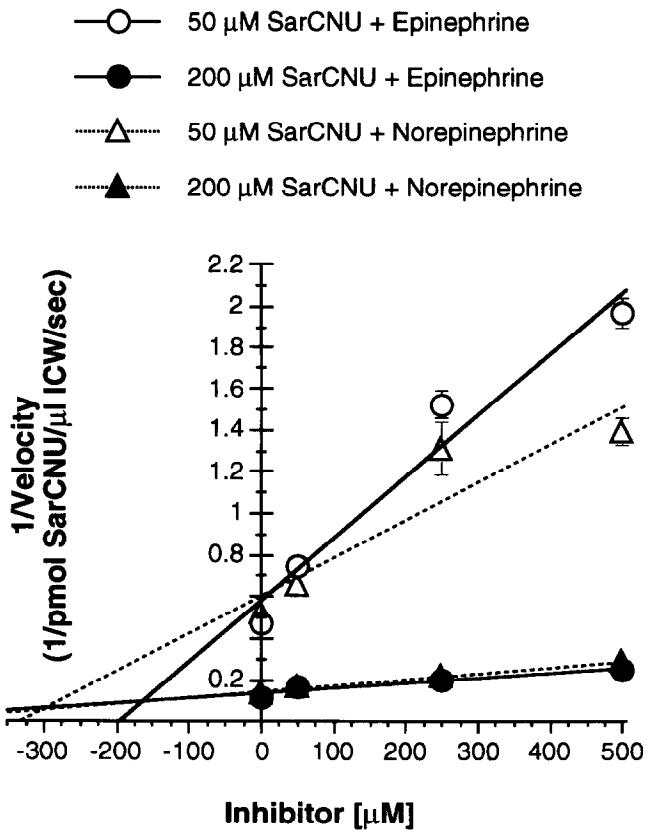


**FIG. 2. Dixon plot of (±)-epinephrine and (±) norepinephrine inhibition of [$^3$H]SarCNU influx. The 2-sec uptakes of [$^3$H]SarCNU, at concentrations of 50 and 200 μM, in medium containing 50–500 μM (±)-epinephrine or (±)-norepinephrine were measured in suspension at 37°, as described in Materials and Methods. The $K_i$ values for the average of all experimental values were 165 and 303 μM for (±)-epinephrine and (±)-norepinephrine, respectively. Points: means of three separate experiments with each drug concentration performed in quadruplicate; bars: SE.**

[$^3$H](−)-norepinephrine at 0 sec was 72 ± 2 and 78 ± 1% for SK-MG-1 and 73% for SKI-1 for 25 μM and 10 mM (−)-norepinephrine, respectively. All results are presented corrected for the recovery at 0 sec. The recovery of intact intracellular [$^3$H](−)-norepinephrine from SK-MG-1 cells was 94 ± 1, 90 ± 1, and 86 ± 1% for 25 μM versus ≥96% for 10 mM, at 5, 10, and 15 sec, respectively. The recovery of intact intracellular [$^3$H](−)-norepinephrine from SKI-1 cells was ≥93% for 25 μM versus 100% for 10 mM, at 5, 10, and 15 sec, respectively. Utilizing ANOVA analysis, there was no significant difference in the percent recovery of intact intracellular drug at 5 sec for each cell line when comparing 25 μM versus 10 mM [$^3$H](−)-norepinephrine. No significant intracellular metabolism of [$^3$H](−)-norepinephrine was observed in either cell line at 0°.

### *Time–course of [$^3$H](−)-Norepinephrine Influx at 0° and 37°*

A time–course of the influx of [$^3$H](−)-norepinephrine, at a 25 μM concentration, in SK-MG-1 and SKI-1 cells is shown in Fig. 3. Following correction for metabolism, the influx of [$^3$H](−)-norepinephrine into both cell lines was linear to 15 sec at 37°. The initial rates of influx were similar for both cell lines at 37°. In contrast, at 0°, influx into SK-MG-1 cells was inhibited significantly compared with 37° ($P$ = 0.009), whereas at 0° influx into SKI-1 cells was not inhibited significantly. Additionally, the initial rate of influx of [$^3$H](−)-norepinephrine at concentrations as high as 10 mM was found to be linear to 15 sec (data not shown). All subsequent kinetic experiments were carried out at 5 sec, to approach the initial rate of influx 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 [31].

### *Kinetics of [$^3$H](−)-Norepinephrine Influx*

In SK-MG-1 cells, as the [$^3$H](−)-norepinephrine concentration was increased from 25 μM to 3.0 mM, the cell to

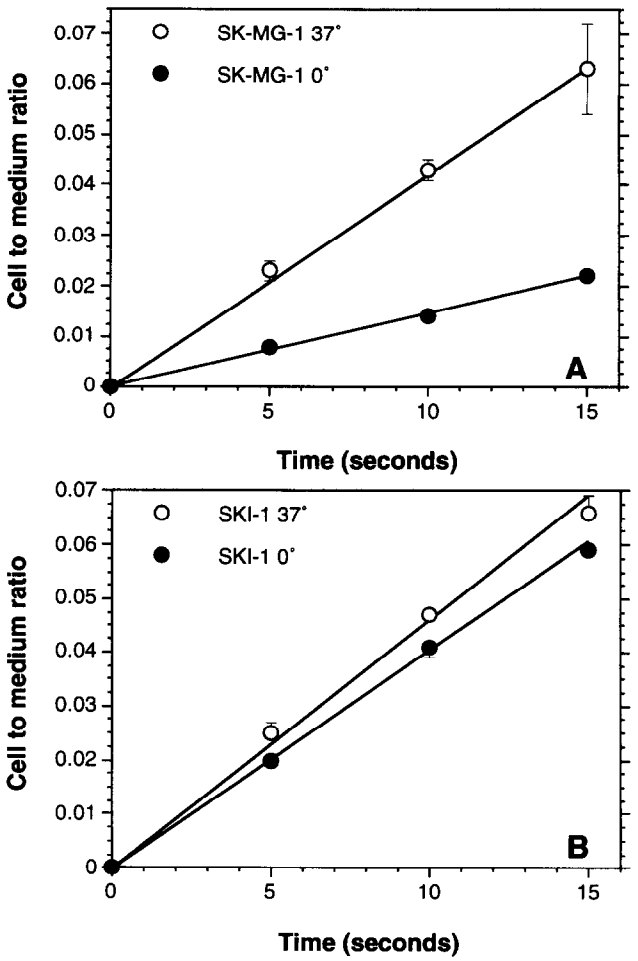


**FIG. 3. Time–course of influx of [$^3$H](−)-norepinephrine, at a 25 μM concentration, by (A) SK-MG-1 and (B) SKI-1 cells in suspension at 0° and 37°. The uptake, in suspension, is expressed as cell:medium distribution ratio, as described in Materials and Methods. The "zero-time" cell:medium ratios were equal to the backgroud contributed by the contamination of the cell pellet with extracellular water space for 0 and 37°. Points: means of at least four separate experiments with each time interval performed in quadruplicate; bars: SE.**

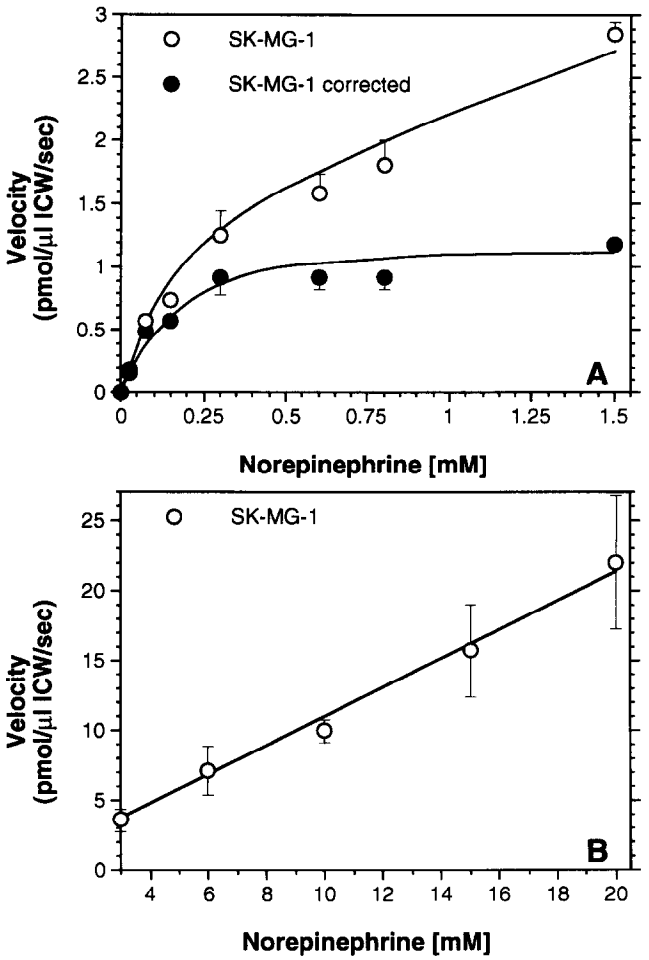


**FIG. 4. Influx of [$^3$H](−)-norepinephrine as a function of concentration. The 5-sec uptakes of SarCNU from (A) 25 µM to 1.5 mM and (B) 3 to 10 mM were measured in suspension at 37°, as described in Materials and Methods. The corrected curve from (A) 25 µM to 1.5 mM represents the velocity minus the diffusional velocity of [$^3$H](−)-norepinephrine in the presence of excess (−)norepinephrine. Points: means of at least four separate experiments with each drug concentration performed in quadruplicate; bars: SE.**

medium ratio, at 5 sec, decreased from 0.038 ± 0.002 to 0.006 ± 0.001. Thereafter, there was no additional decrease in (−)-norepinephrine distribution up to 10 mM (data not shown). The inability to reduce the cell to medium distribution ratio below 0.006 at concentrations greater than 3.0 mM is consistent with non-facilitated diffusion. Therefore, (−)-norepinephrine influx into SK-MG-1 cells is a result of saturability at low concentrations and non-saturability at high concentrations. The relationship between the initial velocity of influx of (−)-norepinephrine into SK-MG-1 and concentration is shown in Fig. 4. When concentrations of (−)-norepinephrine exceeded 3 mM, the velocity of influx into SK-MG-1 could be seen to be linearly dependent on concentration consistent with passive diffusion (Fig. 4B). Regression of the velocities of influx between 3 and 10 mM allowed for estimation of the nonfacilitated diffusion rate to be 1.2 ± 0.2 pmol/µL ICW/sec/mM (−)-norepinephrine. This rate constant was then used to correct the velocity of influx values between 25 µM and 1.5 mM (−)-norepinephrine for passive diffusion (Fig. 4A). The corrected rate of (−)-norepinephrine influx into SK-MG-1 followed Michaelis–Menten kinetics, with non-linear regression analysis of the uncorrected data estimating a $K_m$ of 148 ± 28 µM and a $V_{max}$ of 1.23 ± 0.18 pmol/µL ICW/sec.

In constrast to SK-MG-1 cells, SKI-1 cells did not demonstrate a decrease in the cell to medium ratio at [$^3$H](−)-norepinephrine was increased from 25 µM to 10 mM (ratios of 0.039 ± 0.004 and 0.038 ± 0.005, respectively). The lack of inhibition of the influx of (−)-norepinephrine into SKI-1 cells was consistent with entry of (−)-norepinephrine into these cells via passive diffusion.

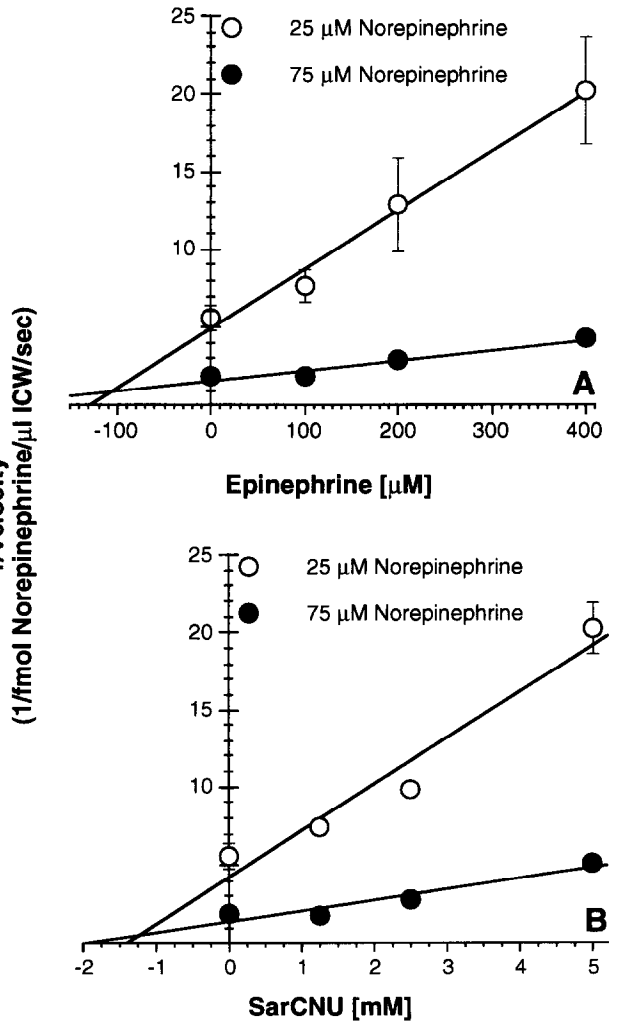


**FIG. 5. Dixon plot of (−)-epinephrine and SarCNU inhibition of [$^3$H](−)-norepinephrine influx. The 5–sec uptakes of [$^3$H](−)-norepinephrine, at concentrations of 25 and 75 µM, in medium containing (A) 50 to 500 µM (−)-epinephrine or (B) 1.25 to 5 mM SarCNU were measured in suspension at 37°, as described in Materials and Methods. The $K_i$ values for the average of all experimental values were 104 µM and 1.2 mM for (−)-epinephrine and SarCNU, respectively. Points: means of three separate experiments with each drug concentration performed in quadruplicate; bars: SE.**

**TABLE 2. Chemical specificity of the influx of norepinephrine and SarCNU in SK-MG-1 cells**

| Compound | Inhibitor | N* | Initital velocity† | % Control‡ |
|---|---|---|---|---|
| 25 μM Norepinephrine | | | fmol/μL ICW/sec) | |
| | Control | 3 | 285 ± 13 | |
| | 10 mM Epinephrine | 3 | 55 ± 17 | 19 ± 5§ |
| | 10 mM Sarcosinamide | 3 | 33 ± 7 | 12 ± 2§ |
| | 100 μM Papaverine | 3 | 75 ± 25 | 26 ± 9§ |
| | 1 μM Disprocynium-24 | 3 | 68 ± 26 | 23 ± 8§ |
| | 100 μmM Cimetidine | 3 | 118 ± 24 | 42 ± 10§ |
| | 10 μM Desipramine | 3 | 287 ± 18 | 100 ± 5‖ |
| 50 μM SarCNU | | | (pmol/μL ICW/sec) | |
| | Control | 4 | 4.43 ± 0.03 | |
| | 10 mM Epinephrine | 4 | 2.34 ± 0.23 | 53 ± 6§ |
| | 10 mM Sarcosinamide | 4 | 2.78 ± 0.03 | 63 ± <1§ |
| | 100 μM Papaverine | 4 | 2.77 ± 0.22 | 59 ± 4§ |
| | 1 μM Disprocynium-24 | 4 | 2.28 ± 0.14 | 52 ± 4§ |
| | 100 μM Cimetidine | 4 | 2.61 ± 0.38 | 64 ± 8§ |
| | 10μM Desipramine | 4 | 4.17 ± 0.29 | 93 ± 7‖ |

SK-MG-1 cells in suspension were examined at 37° for uptake of [$^3$H](−)-norepinephrine (5 sec) or [$^3$H]SarCNU (2 sec) in the absence or presence of the indicated inhibitors, as described in Materials and Methods.

a* Number of individual experiments.

b† Velocity values (mean ± SEM) were not corrected for diffusion.

c‡ Percent difference (mean ± SEM) between control velocity versus velocity in the presence of inhibitor compound.

d§ Significantly different, at 95%, from control velocity and velocity in the presence of desipramine as determined by the Scheffè ANOVA test.

e‖ Not significantly different from control velocity.

### *Dixon Plot Analysis of [$^3$H](−)-Norepinephrine Transport*

In SK-MG-1 cells, the Dixon plot analysis [28] of the effect of increasing concentrations of (−)-epinephrine and SarCNU on the uptake of [$^3$H](−)-norepinephrine, at concentrations of 25 and 75 μM, at 37° is shown in Fig. 5. The influx of (−)-norepinephrine was corrected for nonfacilitated diffusion in all cases. The point of intersection of the two regression lines for (−)-epinephrine (Fig. 5A) and SarCNU (Fig. 5B) was consistent with competitive inhibition of (−)-norepinephrine influx by both compounds. The mean $K_i$ for three independent experiments was found to be 111 ± 7 μM and 1.5 ± 0.2 mM for (−)-epinephrine and SarCNU, respectively. The $K_i$ value determined for (−)-epinephrine was similar to the $K_i$ value determined previously in Dixon plot analysis of [$^3$H]SarCNU uptake with epinephrine inhibition [15]. The $K_i$ value determined for SarCNU was similar to the $K_m$ value for SarCNU transport, determined previously [15].

### *Chemical Specificity of [$^3$H](−)-Norepinephrine and SarCNU Transport*

Neuronal uptake$_1$ and extraneuronal uptake$_2$ inhibitors were tested for the ability to inhibit the influx of [$^3$H]-(−)norepinephrine, at a 25 μM concentration, and [$^3$H]SarCNU, at a 50 μM concentration, into SK-MG-1 cells, under initial rate conditions (Table 2). The pattern of inhibition was similar for both [$^3$H](−)-norepinephrine and [$^3$H]SarCNU with reduction of influx occurring for known uptake$_2$ inhibitors (epinephrine, papaverine, disprocynium-24, sarcosinamide [13], and cimetidine, also an inhibitor of the renal cationic transporter [32]) but not by desipramine, a potent neuronal uptake$_1$ inhibitor.

### *Effect of Ouabain and KCl on Influx*

The transport of norepinephrine has been shown to be dependent on membrane potential; depolarization decreases uptake$_2$ transport of catecholamines [33]. The presence of the $Na^+/K^+$ ATPase inhibitor, ouabain, and excess external KCl are capable of depolarizing membrane potential [27]. Therefore, the initial rate of influx of [$^3$H](−)-norepinephrine, at a 25 μM concentration, into SK-MG-1 cells, at 37°, was examined in the presence of 100 mM KCl, with an equimolar decrease in NaCl, or 0.4 mM ouabain. In three paired experiments, the presence of 100 mM KCl and 0.4 mM ouabain significantly reduced the influx of (−)-norepinephrine by 37 ± 3 and 28 ± 4%, respectively ($P$ = 0.0029 and 0.0026, respectively). In contrast, the influx of [$^3$H]SarCNU, at a 50 μM concentration, into SK-MG-1 cells was not affected significantly by ouabain [15] or by 100 mM KCl (results not shown).

## DISCUSSION

This study was initiated to define the exact mechanism of uptake of SarCNU, an experimental CENU, into the malignant human glioma cell lines SK-MG-1 and SKI-1. Recent studies have suggested that SarCNU enters SKI-1 cells

through a technically non-saturable diffusional influx. However, SarCNU enters SK-MG-1 cells by an epinephrine-sensitive transporter that is not sensitive to metabolic inhibitors or NaCl [15]. In our current investigation, the analysis of the influx of SarCNU into SK-MG-1 cells in the presence of epinephrine and norepinephrine revealed that both compounds are able to inhibit competitively SarCNU transport into SK-MG-1 cells with greater affinity for epinephrine (smaller $K_i$) than norepinephrine. This pattern of inhibition is consistent with the catecholamine uptake$_2$ transporter [34]. The carrier for SarCNU has been shown to be inhibited by the presence of sarcosinamide, under initial rate conditions, which shares a carrier with epinephrine [13]. Sarcosinamide has also been shown to reduce the *in vitro* toxicity of SarCNU against SK-MG-1 cells [12]. The present result suggests that the reduced toxicity of SarCNU in the presence of sarcosinamide is a consequence of reduced accumulation of SarCNU in SK-MG-1 cells over the 60-min exposure period.

Evidence suggests that the sensitivity of SK-MG-1 cells to SarCNU is due to the presence of an epinephrine-sensitive carrier. However, SKI-1 cells do not appear to have this carrier-mediated mechanism [14, 35]. These results were obtained indirectly through the use of [$^3$H]SarCNU. In the present study, [$^3$H](−)-norepinephrine was used to address directly the extraneuronal uptake$_2$ transporter status of SK-MG-1 and SKI-1 cells. [$^3$H](−)-Norepinephrine was used since the transport of catecholamines by uptake$_2$ has been shown to be stereoselective for some substrates (i.e. epinephrine) [36, 37]. The uptake$_2$ transporter is involved in an intracellular metabolizing cascade of norepinephrine and epinephrine via COMT and MAO [21]. Therefore, the ability of both SK-MG-1 and SKI-1 cells to metabolize norepinephrine was investigated. Following 30 min of incubation, there was significant metabolism of norepinephrine in both cell lines. The addition of U-0521, a COMT inhibitor, significantly increased the recovery of intact norepinephrine. However, the addition of pargyline, an MAO inhibitor, did not affect the recovery of intact norepinephrine in either cell line. These results suggest that the primary means of inactivation of norepinephrine in both SK-MG-1 and SKI-1 cells is COMT, consistent with uptake$_2$ transport. Since U-0521, a COMT inhibitor, is known to inhibit uptake$_2$ transport [29, 30], analysis of metabolism without COMT or MAO inhibition was carried out to determine if U-0521 and pargyline were necessary. In the absence of inhibition, there was no significant norepinephrine metabolism at 5 sec in either cell line.

The influx of norepinephrine was linear to 15 sec in both cell lines but only temperature dependent in SK-MG-1 cells. Furthermore, the accumulation of norepinephrine into SKI-1 cells appears to occur only through non-saturable (diffusional) uptake. The accumulation of norepinephrine was saturable in SK-MG-1 cells with a $K_m$ of 148 μM similar to the $K_i$ of norepinephrine for inhibition of SarCNU accumulation. Accumulation of norepinephrine was found to be inhibited competitively by epinephrine and SarCNU in SK-MG-1 cells. Furthermore, Dixon plot analysis of norepinephrine (inhibited with SarCNU and epinephrine) and SarCNU uptake (inhibited with norepinephrine and epinephrine) suggest that SarCNU and the catecholamines are transported by a common carrier in SK-MG-1 cells. Also, the pattern of inhibition of this carrier by various compounds is consistent with the catecholamine uptake$_2$ transporter.

The catecholamine uptake$_2$ transporter which is inhibited by O-methylated catecholamines, corticosteroids, and β-haloalkylamines [38] is clearly different from uptake$_1$, and there is evidence for its presence, *in vivo*, in various sympathetically innervated tissues such as myocardium, salivary glands, and vascular smooth muscle [32]. However, the catecholamine uptake$_2$ transporter has been described in only two cell lines [32, 39]. The presence of uptake$_2$ in astrocytes has been difficult to assess, since uptake of norepinephrine is followed by metabolism by COMT and to a lesser extent MAO [21]. Carrier-mediated uptake of catecholamines has been demonstrated in rodent astrocytes [22, 40]. Therefore, the chemical specificity of norepinephrine and SarCNU transport was examined in SK-MG-1 cells in order to determine the exact nature of the carrier. The extraneuronal uptake$_2$ inhibitors, epinephrine, papaverine, and disprocynium-24, and the renal cationic transport inhibitor, cimetidine, were able to inhibit the saturable portion of the influx of both compounds. Sarcosinamide, the carrier moiety present within the structure of SarCNU, has been shown previously to inhibit influx, consistent with the observation that it shares a common carrier for catecholamines and SarCNU [13]. The theoretical carrier specific transport of norepinephrine and SarCNU calculated from Michaelis–Menten kinetics contributes 85 and 60% of total influx at experimental concentrations, respectively. The inhibition of influx by the uptake$_2$ inhibitors and sarcosinamide was similar to the theoretical values. Furthermore, the neuronal uptake$_1$ transport inhibitor, desipramine, at a concentration significantly greater than the nanomolar $K_i$ necessary for inhibition of uptake$_1$, is unable to inhibit significantly the influx of norepinephrine and SarCNU [41]. The lack of inhibition with desipramine and the inhibition of influx with classic uptake$_2$ inhibitors plus the new highly potent uptake$_2$ inhibitor, disprocynium-24 [42], and the renal cationic inhibitor, cimetidine [32, 39], are consistent with influx of SarCNU into SK-MG-1 cells occurring through the uptake$_2$ transporter.

The driving force of catecholamine uptake$_2$ has been shown to be dependent on membrane potential with depolarization and hyperpolarization leading to decreased and increased accumulation of norepinephrine, respectively [33, 43]. These results have led to the suggestion that it is the positively charged substrate that is transported by the uptake$_2$ transporter [27]. In this study, membrane depolarization was achieved by excess extracellular KCl and the ad-

dition of the $Na^+/K^+$ ATPase inhibitor, ouabain. The depolarization of SK-MG-1 cells significantly inhibited the accumulation of norepinephrine consistent with the previous observations of catecholamine uptake$_2$. However, SarCNU accumulation was not affected significantly by either ouabain [15] or KCl. This would suggest that the SarCNU species transported may not be positively charged, which could account for its reduced affinity for the uptake$_2$ transporter.

Saturable uptake of a non-selective uptake$_2$ substrate has been demonstrated in several primary human gliomas but not characterized [20]. Utilizing [$^3$H]norepinephrine, we have characterized this saturable uptake via inhibition and membrane potential studies. Our results are consistent with [$^3$H]norepinephrine uptake via the catecholamine extraneuronal uptake$_2$ transporter in SK-MG-1 cells. This is the first detailed description of this transporter in human glioma cell lines. Recently, the concentration of norepinephrine and epinephrine in the cerebrospinal fluid of normal human subjects has been shown to be $\leq$2.1 and 2.8 nM, respectively [44]. Furthermore, following stimulation, the release of *in vivo* norepinephrine at selected locus ceruleus target areas in the rat has resulted in an increase in norepinephrine concentrations to a maximum of 2.4 $\mu$M [45]. Plasma concentrations of resting norepinephrine and epinephrine levels are 3 and 0.3 nM, respectively [46]. These relatively low concentrations of norepinephrine and epinephrine would not significantly interfere with the accumulation of SarCNU through the uptake$_2$ transporter.

In summary, the results of this detailed analysis of SarCNU and norepinephrine influx into SK-MG-1 and SKI-1 cells are consistent with increased accumulation of SarCNU in SK-MG-1 cells by the extraneuronal catecholamine uptake$_2$ transporter not detectable in SKI-1 cells. Additionally, this is the first demonstration of the presence of the extraneuronal uptake$_2$ transporter in a human glioma cell line. The presence of the uptake$_2$ transporter is responsible for the selective cytotoxicity of SarCNU in SK-MG-1 cells versus SKI-1 cells [14]. This suggests that the presence of the catecholamine uptake$_2$ transporter in tumor cells will enhance SarCNU toxicity. SarCNU is currently undergoing extensive preclinical studies at the National Cancer Institute in view of potential clinical trials. Evaluation of SarCNU as compared with BCNU in athymic mice bearing human gliomas indicates a superior therapeutic index for SarCNU [47].

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