Characterization of the transport of sarcosinamide chloroethylnitrosourea by the catecholamine extraneuronal uptake$_2$ carrier in human glioma cell lines and its relation to its selective cytotoxicity

by

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Abstract

Previous work demonstrated that influx of (2-chloroethyl)-1-sarcosinamide-1-nitrosourea (SarCNU) may be altered from the known influx of clinically available chloroethylnitrosoureas which occurs through passive diffusion. The objective of this thesis was to determine the exact mode of influx of SarCNU into human glioma cell lines SK-MG-1 and SKI-1 which are sensitive and resistant to SarCNU, respectively. The influx of SarCNU into SK-MG-1 cells was shown to be mediated by a saturable, energy and sodium independent epinephrine sensitive carrier system. Analysis of influx of SarCNU into SKI-1 cells demonstrated a technically non-saturable mechanism of entry consistent with passive diffusion. Steady-state accumulation of SarCNU was demonstrated to be greater in SK-MG-1 cells versus SKI-1 cells at 37°C whereas there was similar accumulation at 22°C. Differences in steady-state accumulation were not attributable to altered metabolism or efflux. Increased accumulation of SarCNU in SK-MG-1 cells at 37°C was identified to be a consequence of an increased initial rate of influx at 37°C in SK-MG-1 cells versus SKI-1 cells with no significant difference at 22°C. Analysis of chloroethylnitrosourea sensitivity revealed that SKI-1 cells were 3 fold resistant versus SK-MG-1 to SarCNU at 37°C but only 2 fold resistant at 22°C with no temperature shift effect on the 2 fold level of resistance to BCNU. A more detailed analysis of the SarCNU carrier involving the analysis of norepinephrine influx revealed that SarCNU influx into SK-MG-1 cells occurs through the extraneuronal catecholamine uptake$_2$ transporter which is not detectable in SKI-1 cells. This is the first direct demonstration of the presence of the uptake$_2$ transporter in a human glioma cell line. These findings suggest that increased sensitivity of SK-MG-1 cells to SarCNU is secondary to enhanced accumulation of SarCNU mediated via the uptake$_2$ transporter which is not detectable in SKI-1 cells.

Résumé

Des études ultérieures ont démontré que le transport du (2-chloroéthyl)-1-sarcosinamide-1-nitrosoruée (SarCNU), un nouveau médicament ayant prouvé expérimentalement une sélectivité vis-à-vis des tumeurs d'origine cérébrales, est distinct de celui d'autres chloroethylnitrosourées utilisés cliniquement. L'objectif de cette thèse était d'étudier le mécanisme de transport du SarCNU dans deux lignées cellulaires d'origine gliales, SK-MG-1 et SKI-1, et qui sont sensibles et résistantes au SarCNU, respectivement. Nous avons démontré que le transport du SarCNU dans les cellules SK-MG-1 est saturable, il est indépendant de l'énergie et du sodium et sensible à l'épinéphrine. A l'inverse, le transport du SarCNU dans les cellules SKI-1 est non saturable et semble se faire par diffusion passive. A une température de 37°C, l'accumulation maximale du SarCNU est plus élevée dans les cellules SK-MG1 que dans les cellules SKI-1, alors qu'à 22°C l'accumulation du SarCNU est identique dans les deux lignées. La différence dans l'accumulation maximale ("steady-state") entre les deux lignées n'est pas attribuée à une altération du métabolisme ou du reflux du médicament vers le milieu extracellulaire. Cependant, l'augmentation de l'accumulation maximale du SarCNU dans les cellules SK-MG1 à 37°C a été identifiée comme une conséquence de l'augmentation de la cinétique initiale de l'accumulation ("influx") dans les cellules SK-MG1 par rapport à cellules SKI-1. Cette différence disparaît lorsque les cellules sont maintenues à 22°C. A 37°C, les cellules SKI-1 sont 3 fois plus résistantes au SarCNU que les cellules SK-MG1 alors qu'elles sont seulement 2 fois plus résistantes que les cellules SK-MG1 à 22°C. Cette différence n'est pas observée avec le bis-chloroéthylnitrosourée (BCNU). Une étude détaillée a révélé que le transporteur du SarCNU est similaire à celui de la norépinéphrine. En effet, l'accumulation de SarCNU dans SK-MG-1 se fait par l'intermédiaire du transporteur 2 extraneural des catécholamines qui n'est pas détecté dans les cellules cellules SKI-1. Notre étude constitue la première évidence de la présence du transporteur 2 dans des cellules gliales humaines. Nos résultats suggèrent que l'augmentation de la sensitivité des SK-MG-1 au SarCNU est secondaire à

une augmentation de l'accumulation via le transporteur 2, à l'inverse des cellules SKI-1 ou ce transporteur semble être absent.

This work is dedicated to my father, Robert

(WHERE LIFE ENDED ALL TOO SOON)

and to my wife, Karen, and daughter, Alycia.

(WHERE LIFE BEGINS).

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It seemed as it would never end but we finally made it!

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PREFACE

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

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Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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perfectly clear the responsibilities of all authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**

Chapter 2:

Noë, A. J., Malapetsa, A., and Panasci, L. C. 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, Molecular Pharmacology *44:* 204-209, 1993.

Chapter 3:

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Chapter 4:

Noë, A. J., Marcantonio, D., Barton, J., Malapetsa, A., Panasci, L. C. Characterization of the catecholamine extraneuronal uptake2 carrier in the human glioma cell lines SK-MG-1 and SKI-1 in relation to SarCNU selective cytotoxicity, Biochemical Pharmacology, *51:* 1639-48, 1996.

The candidate was responsible for all transport work and preparation of cell lysates for analysis of metabolism. Some metabolism analysis was performed by other investigators. This is acknowledged by including their names in each paper. Their contributions are described in the preface to each chapter.

ABBREVIATIONS

ACNU	:	1-[(4-amino-2-methylpyrimidin-5-yl)methyl]-3-(2-chloroethyl)-3-nitrosourea hydrochloride
BCH	:	2-aminoendobicyclo(2,2,1)-heptane-2-carboxylic acid
BCO	:	3-aminoendobicyclo(3,2,1)octane-3-carboxylic acid
BCNU	:	1,3-bis-(2-chloroethyl)-1-nitrosourea
BCyNU	:	1,3-biscyclohexyl-1-nitrosourea
benzylamine	:	phenylmethanamine
BSO	:	buthionine sulfoximine
BTCG	:	Brain Tumor Cooperative Group
CCNU	:	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CENU	:	chloroethylnitrosourea
CFU	:	colony formation unit
Chlorozotocin	:	2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose
cis-2-OH-CCNU	:	1-(2-chloroethyl)-3-(cis-2-hydroxycyclohexyl)-1-nitrosourea
cisplatin	:	cis-diamminedichloroplatinum(II)
CNS	:	central nervous system
CNU	:	N-(2-chloroethyl)-N-nitrosourea, or 1-(2-chloroethyl)-1-nitrosourea
COMT	:	catechol-O-methyltransferase
decynium22	:	1,1'-diethyl-2,2'-cyanine
disprocynium24	:	1,1'-diisopropyl-2,4'-cyanine

D,L-NAM	:	D,L-2-Amino-7-bis[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic acid
DOMA	:	3,4-dihydroxymandelic acid
DOPEG	:	3,4-dihydroxyphenylglycol
D'PBS	:	Dulbecco's incomplete phosphate-buffered saline
ENU	:	1-ethyl-1-nitrosourea
ERCC	:	excision-repair cross complementing
FCNU	:	1-(2-fluoroethyl)-3-cyclohexyl-1-nitrosourea
GSH	:	glutathione, γ-glutamylcysteinylglycine
GST	:	glutathione-S-transferase
HN1	:	dimethyl 2-chloroethylamine
HN2	:	nitrogen mustard, mechlorethamine
HN2-OH	:	hydrolyzed nitrogen mustard
IC_{50}	:	50% inhibitory concentration
ICW	:	intracellular water space
ISCL	:	interstrand cross-link
K_i	:	inhibition constant, which represents the concentration of an inhibitor where the velocity of a substrate is equal to 50% of V_{max} for carrier-mediated transport or total transport if diffusion is not subtracted from uptake values
K_m	:	Michaelis-Menten constant, which represents the concentration of a substrate where the velocity is equal to 50% of V_{max} for carrier-mediated transport
MDR	:	multi-drug resistant
melphalan	:	l-phenylalanine mustard, L-PAM, Alkeran

MGMT	:	O^6-methylguanine-DNA methyltransferase
MAO	:	monoamine oxidase
MAT	:	vesicular monoamine transporter
MeAIB	:	2-(methylamino)-isobutyrate
MNNG	:	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	:	1-methyl-1-nitrosourea
MOPEG	:	3-methoxy-4-hydroxyphenylglycol
MPP^+	:	1-methyl-4-phenylpyridinium
NE	:	norepinephrine
NET	:	norepinephrine $uptake_1$ transporter
NMN	:	normetanephrine
OMI	:	3-O-methylisoprenaline
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
PNS	:	peripheral nervous system
PPC	:	peak plasma concentration
RTOC	:	renal transport of cations
SarCNU	:	(2-chloroethyl)-3-sarcosinamide-1-nitrosourea
sarcosinamide	:	N-methylglycinamide
serotonin	:	5-hydroxytryptamine
SRB	:	sulforhodamine B
$Uptake_1$	:	neuronal catecholamine transport
$Uptake_2$	:	extraneuronal catecholamine transport
VMA	:	3-methoxy-4-hydroxymandelic acid
V_{max}	:	the maximal velocity of carrier-mediated transport for a substrate

xylamine : N-2-chloroethyl-N-ethyl-2-methyl-benzylamine

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

Chloroethylnitrosoureas (CENUs) are an important class of alkylating anticancer chemotherapy drugs with important single agent effectiveness in the treatment of malignant human gliomas. The mechanism of cytotoxicity of this class of drugs is thought to be a consequence of the generation of electrophilic alkylating derivatives which react with nucleophilic centers on DNA bases to form a covalent bond. This covalently attached alkyl product then reacts with the opposite strand of DNA to form a lethal interstrand cross-link. The CENUs are currently the most effective single agent adjuvant therapy in the treatment of gliomas. However, the effectiveness of treatment of gliomas with CENUs is compromised by delayed cumulative myelosuppression and an apparent innate resistance of brain tumors to this class of drugs. Current techniques that have been developed to circumvent myelosuppression and resistance to CENUs involve the delivery of increased concentrations of CENUs to the site of glioma infiltration. The local delivery of CENUs to the brain is complicated by increased neural toxicity. Thus, the diagnosis of glioblastoma multiforme carries a poor prognosis with little hope for survival greater than 2 years. The development of alternative single agent or combined agent therapies for the treatment of gliomas with increased effectiveness and reduced systemic and local toxicity is therefore a priority as concerns the effective medical control of malignant glioma.

(2-Chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) is a derivative of CENU that has an amino acid amide, sarcosinamide, attached at its N-3 atom. It is unique from classical CENUs in that it has a methyl group attached at the N-3 position (tertiary N) instead of a

hydrogen atom. The presence of the methyl group is apparently responsible for the increased stability of SarCNU at physiological pH and its reduced systemic toxicity in mice. Previous work in Dr. Panasci's laboratory has demonstrated that SarCNU is more effective in the *in vitro* treatment of primary human gliomas than 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and is less toxic to human bone marrow. Moreover, it was demonstrated through indirect means that SarCNU may be transported into human glioma cell lines, SK-MG-1 and SKI-1, as demonstrated by a competitive inhibition of SarCNU for the transport of sarcosinamide. Indications are that SarCNU is recognized by the same system that can transport sarcosinamide and epinephrine. SKI-1 cells have a distinguishable reduction in affinity for SarCNU compared to SK-MG-1 cells. Furthermore, SarCNU sensitive SK-MG-1 cells accumulate greater amounts of SarCNU intracellularly as compared to the SarCNU resistant SKI-1 cells. The accumulation of SarCNU intracellularly was found to correlate with levels of the cytotoxic interstrand cross-linking of DNA within both cell lines. It therefore seems possible that a SarCNU transport system may mediate sensitivity to the compound. Interestingly, analysis of the ability of sarcosinamide to reduce the *in vitro* cytotoxicity of SarCNU has suggested an apparent involvement of a transport system in the cytotoxicity of SarCNU against SK-MG-1 cells. The ability to be recognized by a transport system has not been demonstrated for the current clinically available CENUs, which enter cells by passive diffusion. These data strongly suggested that the addition of sarcosinamide to a CENU has generated a novel CENU that may be transported into human glioma cells and that this may account for the increased effectiveness of SarCNU versus BCNU against gliomas.

The primary goal of this thesis was to characterize fully the transport of SarCNU into human glioma cell lines SK-MG-1 and SKI-1. SarCNU became available in a radiolabeled form prior to the initiation of this project which allowed the direct assessment of the its transport into SK-MG-1 cells and SKI-1 cells. I initially investigated the transport of SarCNU into sensitive SK-MG-1 cells (Chapter 2). The second stage of my project was to assess if differences in SarCNU transport between SK-MG-1 cells and SKI-1 cells are responsible for the increased effectiveness of SarCNU against SK-MG-1 cells (Chapter 3). Following this analysis, it was necessary to address the physiological identity of the SarCNU transport system in order to fully characterize the involvement of its transporter in altering sensitivity to SarCNU (Chapter 4). This thesis deals directly with the transport of SarCNU and its potential involvement in increased effectiveness in the therapy of malignant human glioma.

1 CHLOROETHYLNITROSOUREAS

1.1 Chemical reactivity of chloroethylnitrosoureas

The chloroethylnitrosoureas (CENUs) are a class of alkylating anti-cancer chemotherapeutic agents that are utilized to treat brain tumors and a variety of systemic malignancies [1, 2]. Haloethylnitrosoureas were originally developed after it was noted that N-methyl-N'-nitro-N-nitrosoguanidine demonstrated antitumor activity against L1210 cells in mice [3]. Most haloethylnitrosoureas with antitumor activity are N-1-nitroso-, N-1-(2-chloroethyl) compounds which decompose at physiological pH to generate a product capable of alkylating DNA, RNA and proteins [4, 5]. The parent compound of CENUs is (2-chloroethyl)-1-nitrosourea which is modified by the addition of a structurally distinct side chain at the N-3 position to give rise to chemically distinct alkylating agents. Clinically available CENUs are lipophilic and stable in serum free media at a pH of 4 [6, 7]. At physiological pH, the clinically available CENUs decompose in a rate limiting step catalyzed by bases and possibly some nucleophiles (Fig. 1.1) [8-13]. The portion of the compound attached to the N-1 atom forms a chloroethyldiazohydroxide intermediate which can undergo low energy SN2 reactions (bimolecular) of low selectivity [12] or can rearrange with loss of nitrogen to yield the 2-chloroethylcarbonium ion, a chloronium ion or a 1-chloroethylcarbonium which are capable of alkylation through an SN1 (unimolecular) mechanism [4, 12]. The 1-chloroethylcarbonium ion would not be capable of delivering a 2-chloroethyl group for alkylation. More recently, the fact that no significant DNA alkylation is observed at pH 5.0 by nitrosoureas suggests that alkylation of DNA may be due to the generation of the chloroethyldiazohydroxide intermediate of CENU

BCNU

1, 3-bis-(2-chloroethyl)-1-nitrosourea

SarCNU

(2-chloroethyl)-3-sarcosinamide-1-nitrosourea

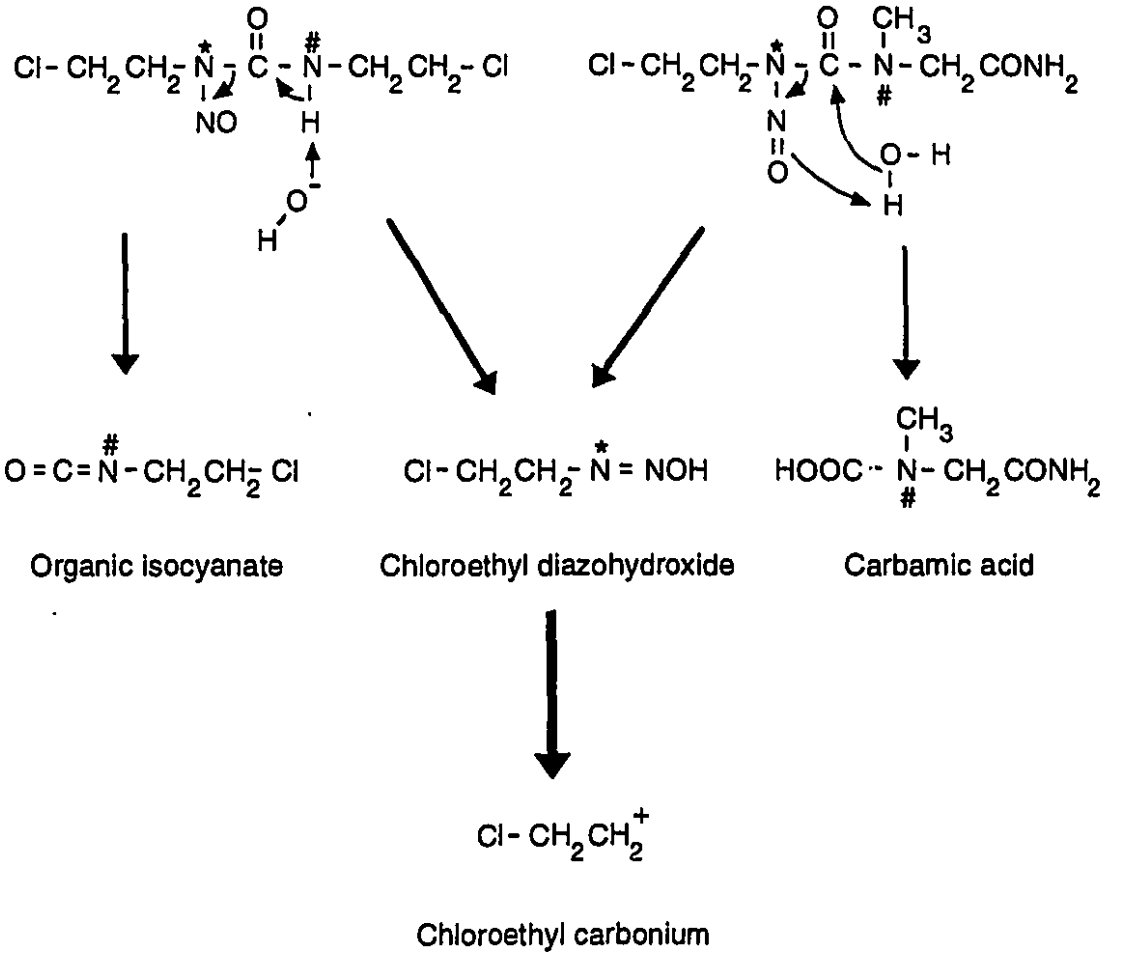


Figure 1.1 Proposed mechanism of degradation of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and (2-Chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) SarCNU. The N-1 atom is represented by the asterisk (*) and the N-3 atom is represented by the pound sign (#). The chloroethyldiazohydroxide can either react via a SN2 mechanism or degrade to chloroethylcarbonium which reacts via a SN1 mechanism.

degradation [14]. This result is consistent with an SN2 mechanism of CENU alkylation through the chloroethyldiazohydroxide. Furthermore, analysis of the electron influences on DNA alkylation have suggested that the small ionization potential of a guanosine base compared to phosphate is consistent with reactivity data indicating that DNA and RNA are subject to electrophilic SN2 attack by 1-methyl-1-nitrosourea (MNU) [15]. The portion of the compound attached to the N-3 atom forms an isocyanate capable of carbamoylation of the ε-amino group of lysine, the α-amino groups of amino acids and the terminal amino groups of proteins without significant reaction with DNA or RNA [16]. While the chloroethyldiazohydroxide ion is identical for all CENUs, the organic isocyanate differs with respect to the side group attached to the N-3 atom. The differences in the N-3 side chain lead to altered levels of stability of the CENU at physiological pH but also to different carbamoylating potentials. (2-Chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) is a novel analog of CENU that has a N-methylglycinamide (sarcosinamide) attached at the N-3 atom (Fig. 1.1) [17]. The chemical half-life of SarCNU has been reported to be approximately 330 minutes at pH 7.4 and 37°C which represents the most stable form of CENU identified to date. A more recent analysis of the stability of SarCNU has demonstrated that at room temperature its chemical half-life is 24 hours in phosphate buffered saline under which conditions other nitrosoureas have half-lives of approximately 8 hours [18]. The stability of SarCNU has been proposed to be a consequence of the presence of a tertiary amino group due to the presence of a methyl group on the N-3 atom which imposes a steric hindrance to the usual route of base catalyzed hydrolysis. The proposed pathway of

decomposition of SarCNU involves hydrolysis at the carbonyl carbon of the nitrosourea portion of SarCNU which results in the generation of the chloroethyldiazohydroxide ion from the N-1 atom and carbamic acid instead of isocyanate from the N-3 atom (Figure 1.1). The carbamic acid can decompose further to yield carbon dioxide and sarcosinamide. Recently, it has been demonstrated that SarCNU undergoes specific acid and base catalysis below pH 3.0 and above pH 7.0, respectively [18].

As the case with many antitumor agents, CENUs act through the formation of electrophilic derivatives, the electron deficient 2-chloroethylcarbonium ion (SN1) and the chloroethyldiazohydroxide (SN2) for CENUs , which then react with covalently nucleophilic centers that are present within the cell. The nitrosoureas, as demonstrated through the use of 1-ethyl-1-nitrosourea (ENU) and MNU, are of low nucleophilic selectivity [19]. The reaction rates of alkylating agents with nucleophilic centers generally increases in the order O (presented as carboxylate ions and hydroxyl groups) < N (presented as amino groups and rings) < S (presented as thiol and thioether sulphurs) which are the main nucleophilic centers in biological macromolecules [20]. The nucleophilicity of the N7 on guanine is greater than the O^6 atom of guanine. CENUs demonstrate an increased ratio of O^6/N7-alkylation indicating a low nucleophilic selectivity [19].

It has been demonstrated that CENUs are capable of alkylating many sites on DNA and RNA bases [5]. The identification of DNA-CENU reaction products of DNA isolated from treated cells has been carried out for ethylnitrosourea (ENU) and N-(2-chloroethyl)-N-nitrosourea (CNU) [21-23]. The primary DNA modifications found in a sensitive human glioma cell line treated with CNU were, in order of decreasing percentage: (a)

chloroethyl/ethylphosphotriesters (approximately 45%), (b) N7-(2-hydroxyethyl)guanine (approximately 20%), (c) N7-(2-chloroethyl)guanine (approximately 20%), (d) 4 base modifications consisting of N3-(2-hydroxyethyl)deoxyuridine, N1-(2-hydroxyethyl)deoxyguanosine, N7-(2-chloroethyl)deoxyguanosine and 1,2-(diguan-7-yl)ethane (approximately 5%) and (e) 1-(N3-deoxycytidyl),2-(N1-deoxyguanosinyl)ethane (approximately 3%) all of which account for approximately 94% of total modified DNA [23]. The O^4- and O^2-modified thymine, O^2-modified deoxycytidine, O^6-modified deoxyguanosine and 3-modified deoxyadenosine products characterized in the DNA of cells treated with ENU are of extremely low incidence in CNU modified DNA [21, 22]. The fact that modifications of oxygen by ENU contribute to approximately 23% of total DNA alkylation lend support to the fact that nitrosoureas show low nucleophilic selectivity [21, 22].

The apparent absence of oxygen atom alkylation by CNU may be, in part, due to the fact that the initial chloroethyl alkylation at the O^6-atom of deoxyguanosine in DNA is thought to be too reactive to isolate conventionally. However, fluoroethyl alkylation of DNA with 1-(2-fluoroethyl)-3-cyclohexyl-1-nitrosourea (FCNU) can produce O^6-(2-fluoroethyl)guanosine suggesting that haloethylnitrosoureas with stronger leaving groups, such as the chloride ion, can alkylate guanine at the O^6 atom [24]. Interestingly, the O^6-(2-fluoroethyl)guanosine can be hydrolyzed to N1-(2-hydroxyethyl)guanosine which is thought to occur through the cyclic intermediate N1,O^6-ethanoguanine which has undergone intramolecular rearrangement from O^6-(2-fluoroethyl)guanosine. In the absence of hydrolysis, O^6-haloethylation is thought to lead to an interstrand cross-link (ISCL) between adjacent DNA strands through the

N1,O^6-ethanoguanine intermediate to produce a 1-(N3-deoxycytidyl),2-(N1-deoxyguanosinyl)ethane cross-link (Fig. 1.2) [25]. The presence of the N1,O^6-ethanoguanine cross-link intermediate is strongly supported by the fact that reaction of human O^6-methylguanine-DNA-methyltransferase (MGMT), a DNA repair protein specific for O^6-alkylguanine, with CENU treated DNA leads to a covalent linkage between DNA and MGMT with the structure of N1-(guan-1-yl)-2-(cystein-S-yl)ethane (Fig 1.2) [26]. It has also been proposed that O^6-alkylation of DNA by CNU to produce O^6-(2-chloroethyl)guanine can cyclize to form an O^6-N7-ethanoguanine which can then be hydrolyzed to produce N7-(2-hydroxyethyl)guanine [27]. These methods of intramolecular rearrangement of O^6-(2-chloroethyl)guanine identified can account for the apparent lack of oxygen atom alkylation by CNU.

1.2 Mechanism of chloroethylnitrosourea antitumor activity

Alkylation of DNA by CENUs has been widely accepted as the mechanism of antitumor activities since CENU compounds with reduced carbamoylation activity retain full antitumor activity [28], whereas nitrosourea compounds devoid of alkylating activity show no antitumor activity [29]. A correlation between the alkylating activity and effective antitumor activity has been demonstrated for CENUs [28, 30, 31]. Furthermore, it has been well documented that alkylation and subsequent ISCL formation is a critical mechanism by which CENUs mediate tumor cell kill [25, 32, 33].

Carbamoylation by CENUs involves the covalent attachment of an isocyanate to the ε-amino group of lysine, the α-amino groups of amino acids or the terminal amino groups of proteins [30, 34, 35]. Although

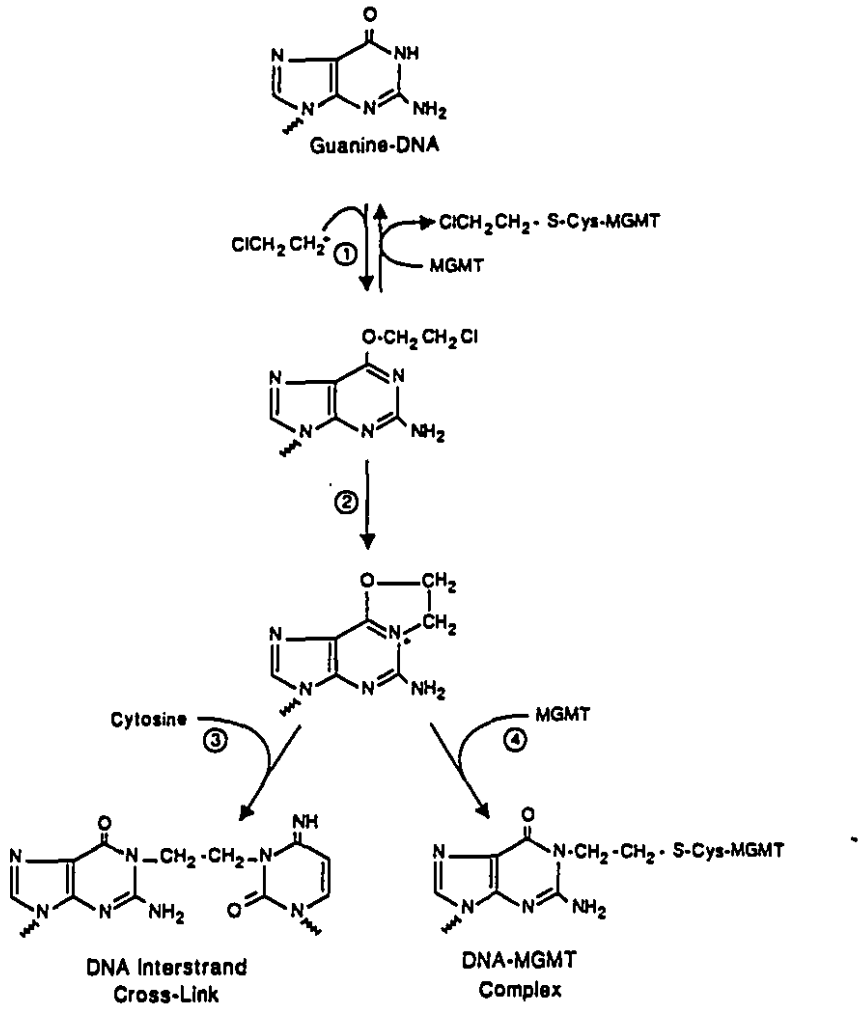


Figure 1.2 Proposed mechanism for CENU induced formation of the 1-[N^3-deoxycytidyl],2-[N^1-deoxyguanosinyl]-ethane interstrand DNA cross-link or the DNA-MGMT complex from a common intermediate. STEP 1 is where the chloroethylcarbonium ion attacks the O^6 position of deoxyguanosine. STEP 2 is susceptible to action of MGMT or rearranges to the N1,O^6-ethanoguanine intermediate. STEP 3 is where the N1,O^6-ethanoguanine intermediate can rearrange with an attack on the opposite strand deoxycytidine to form 1-[N^3-deoxycytidyl],2-[N^1-deoxyguanosinyl]-ethane. STEP 4 involves the action of MGMT on the N1,O^6-ethanoguanine intermediate to form a covalent link between MGMT and DNA through the N1-(guan-1-yl)-2-(cystein-S-yl)ethane link.

carbamoylation does not seem to play a major role in the antitumor activities of CENUs [28, 31, 36], it has been demonstrated to be involved in the inhibition of ligase in excision repair [37], the inhibition of strand rejoining in DNA repair [38, 39], the inhibition of chymotrypsin [35], the inhibition of RNA processing [40], and inactivation of glutathione reductase [41]. Increased carbamoylating activity of CENUs have also been associated with the inhibition of the strand rejoining step associated with DNA repair of drug and X-ray induced strand breaks [42], presumably via the inhibition of DNA ligase. The effect of strong carbamoylating activity is revealed by BCNU and 1,3-biscyclohexyl-1-nitrosourea (BCyNU) which demonstrate therapeutic synergism, *in vitro*, with ionizing radiation [29, 43]. Enhancement of radiation cytotoxicity has little to do with alkylation, since BCyNU, a strong carbamoylating nitrosourea which possesses no alkylating activity, was able to mediate increased sensitivity to radiation [29]. The inhibition of DNA repair by nitrosoureas does not appear to be complete but instead the effect appears to be a consequence of a slowing of the strand rejoining step in repair [39].

Lack of involvement of carbamoylation in the antitumor activity of CENUs has been demonstrated by 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose (chlorozotocin) and 1-(2-chloroethyl)-3-(cis-2-hydroxycyclohexyl)-1-nitrosourea (cis-2-OH-CCNU) which possess weak carbamoylating activity but are effective antitumor agents [28, 42]. Additionally, CNU, which exhibits no biological carbamoylation, displays significant antitumor activity [44]. Conversely, BCyNU which does not have alkylating activity but is a strongly carbamoylating nitrosourea shows little antitumor activity [29].

The molecular mechanism(s) by which the CENUs mediate antitumor activity are not completely elucidated at this time. However, the general consensus is that CENUs mediate their cytotoxic affect through alkylation and subsequent intra- and interstrand cross-linking of DNA. Interstrand cross-linking of DNA is most often associated with the antitumor activity of CENUs. The general scheme of the production of this cross-link occurs in a two step reaction where the chloroethylcarbonium ion, generated by decomposition of CENUs, attacks a nucleophilic site on one strand of DNA thereby enabling a second attachment to a nucleophilic site on the adjacent DNA strand. The initial alkylation reaction is rapid, the majority of which occurs within the first hour, leading to a chloroethylated product [45]. The second step in cross-link formation involves the displacement of a chloride ion by a nucleophilic site on the opposite strand or the N-1 of the same guanine with subsequent covalent linkage between the DNA strands. This second step occurs over an extended period of time, with maximum cross-links observed between 6 and 9 hours, and does not require the presence of an intact CENU [45]. This scheme was originally worked out with purified DNA following treatment with several CENUs and one fluoroethylnitrosourea with which it was proposed that the ISCL rendered DNA insensitive to alkaline denaturation. Subsequent to this initial observation, it was determined that the formation of the cross-link correlated with CENU-induced antitumor toxicity [33, 46]. Additionally, a correlation between cytotoxicity and sister chromatid exchange for cross-linking alkylating agents has strongly suggested that the formation of ISCLs by CENUs are the initiating event in the process of

cytotoxicity [47-49]. In fact, human glioma cell lines resistant to CENUs have fewer DNA ISCLs formed than in sensitive cells [50].

The identity of the CENU mediated ISCL has been investigated over the years. Characterization of the CENU mediated alkylation and cross-links was first carried out on isolated DNA. The low nucleophilic selectivity and thus chemical reactivity of nitrosoureas was first investigated with the use of ENU ([21, 22] and discussed above). The first attempt to characterize CENU mediated cross-links identified a monoadduct N7-(2-chloroethyl)guanine and a cross-link of 1,2-(diguan-7-yl)ethane [51]. Considering the low selectivity of ENU, it was surprising that an O-alkylated CENU mediated product was not isolated. The procedure used to isolate the CENU reaction products utilized controlled depurination of DNA and since alkylation at O^6-guanine is incapable of labilizing the glycosidic bond [52] it is not surprising that O-alkylated products were not characterized. The 1,2-(diguan-7-yl)ethane cross-link was originally thought to originate from chloroethylation at the N7 position of guanine [51]. However, it has also been proposed that O^6-chloroethylation of DNA by a CENU to produce O^6-(2-chloroethyl)guanine can intramolecularly rearrange to form a cyclized O^6-N7-ethanoguanine intermediate which would then lead to either depurination or the formation of a 1,2-(diguan-7-yl)ethane cross-link [27]. The 2-carbon bridge in the 1,2-(diguan-7-yl)ethane cross-link is probably not sufficiently long enough to reach between two guanines in opposite strands within the classical double helix configuration. However, local distortion of the DNA helix upon the first chloroethylation may denature the structure sufficiently to allow intrastrand cross-links. Following the identification of the 1,2-

(diguan-7-yl)ethane cross-link, a new procedure of isolating CENU alkylation products from treated DNA allowed the identification of a 1-(N3-deoxycytidyl),2-(N1-deoxyguanosinyl)ethane cross-link which would meet the steric limitations of a 2 carbon bridge between a guanine:cytosine base pair within the DNA double helix [25]. The proposed mechanism of formation of the ISCL involves an initial chloroethyl alkylation at the O^6-atom following by an intramolecular rearrangement through a N1,O^6-ethanoguanine intermediate with subsequent nucleophilic replacement of the N-3 of cytosine for the O^6-atom of guanine (Fig 1.2). This mechanism accounts for the lag between the initial alkylation of DNA and the formation of DNA ISCLs [53]. The proposed initial alkylation of the O^6-atom of guanine is consistent with the fact that cells which are capable of removing O^6-methylguanine monoadducts and by extension O^6-alkylguanine monoadducts form fewer ISCLs than cells which do not possess the repair phenotype [33, 48]. Additionally, the 1-(N3-deoxycytidyl),2-(N1-deoxyguanosinyl)ethane ISCL has been characterized in CENU sensitive and resistant human glioma cell lines and was demonstrated to be decreased in the CENU resistant cell which possessed a greater quantity of MGMT and ability to repair O6-alkylguanine lesions [23].

Subsequent investigations have provided evidence for the existence of the N1,O^6-ethanoguanine intermediate following treatment of DNA with CENUs. For instance, it has been demonstrated that an O^6-(2-fluoroethyl)guanosine monoadduct, thought to be more stable than the proposed O^6-(2-chloroethyl)guanosine monoadduct, is formed following treatment of DNA with FCNU, which, upon hydrolysis leads to N1-(2-hydroxyethyl)guanosine [24]. The formation of N1-(2-

hydroxyethyl)guanosine lends credence to the existence of the proposed N1,O^6-ethanoguanine intermediate. Further support for the existence of the N1,O^6-ethanoguanine cross-link intermediate comes from the demonstration of a reaction leading to a covalent attachment between human MGMT, a DNA repair protein specific for O^6-alkylguanine, and DNA [54]. The attachment has subsequently been identified as a N1-(guan-1-yl)-2-(cystein-S-yl)ethane link between MGMT and DNA [26]. The authors noted that the rate of decay of the precursor for ISCLs following treatment of DNA with CENUs was the same as the rate decay of the ability to form the N1-(guan-1-yl)-2-(cystein-S-yl)ethane link. Moreover, the half-life of the ability to form ISCLs, which are proposed to occur through the N1,O^6-ethanoguanine intermediate, is consistent with the slow rate of formation of ISCLs observed in cells [26].

The consequence of the formation of an intra- or interstrand cross-link would be the disruption of double helical structure of DNA with subsequent interference of replicative and transcriptional events necessary for the survival of cell. Support for this conclusion comes from the fact that treatment of cells with CENUs leads to a prolonged S phase and G2 arrest within the cell cycle [55]. Cisplatin, an antitumor agent which leads to intrastrand cross-links between N7 atoms of adjacent guanine bases [56], is also capable of inhibiting DNA synthesis [57, 58] and of arresting cells in the G2 phase of the cell cycle [59]. Furthermore, despite the different structures and reactivities of cisplatin and CENUs, a shared blocking lesion for primer extension occurs at the intrastrand cross-link between adjacent guanines through the N7-atom [60]. The technique utilized was not able to distinguish between intrastrand cross-links and ISCLs, therefore the extent of

shared lesions between cisplatin and CENUs is not known. Additional evidence suggests that regions of guanine repeats, which are often found within upstream regulatory sequences important for transcription of genes, are preferentially alkylated by CENUs, as concerns guanine-N7-alkylation [14, 60].

Evidence also exists that other lesions besides ISCLs may be responsible for some of the cytotoxicity associated with CENUs. For instance, the alkylating agents MNU, streptozotocin and ENU which are unable to form DNA cross-links are cytotoxic to cells, although much less so compared to cross-linking CENUs [48, 61, 62]. Furthermore, several *in vitro* studies have demonstrated that O^6-methyl- and O^6-alkylguanine monoadduct lesions lead to ambiguous base pairing properties between hydrogen bonding base pairs which can cause miscoding during replication by bacterial and bacteriophage DNA polymerases [63-65]. Indeed, the presence of O^6-methylguanine within a guanine:cytosine base pair has been demonstrated to lead to primarily guanine:cytosine → adenine:thymidine transitions within human cells, which is consistent with the miscoding potential of O^6-methylguanine [66, 67]. The guanine:cytosine → adenine:thymidine transition has been demonstrated to form point mutations causing expression of a mutant phenotype of an actively expressed gene [66]. Finally, there are bacterial strains that are capable of removing O^6-alkylguanine lesions via bacterial MGMT that are still sensitive to CENUs, suggesting that lesions other than ISCLs may be responsible for CENU toxicity [68]. A similar situation has been demonstrated in DNA-excision repair deficient Chinese hamster ovary cells which have been transfected with MGMT [69]. Although expression of MGMT increased resistance of these cells to CENU, the level of

sensitivity compared to a DNA-excision repair proficient CHO cell line transfected with MGMT was similar to the level prior to transfection. These results suggest that there are other lesions besides O^6-chloroethylguanine mediated ISCLs that are cytotoxic to mammalian cells.

2 BRAIN TUMORS

2.1 Malignant Astrocytomas

Astrocytes are the stellate supporting cells of the brain and spinal cord and are thought to be the progenitors of most glioblastomas since glial fibrillary acidic protein (GFAP, an astrocyte marker) can be identified in most tumors [70]. In the United States, malignant gliomas represent 1% of all adult cancers and account for approximately 2.5% of all cancer deaths [71]. Brain tumors are the second most common cancer after leukemia and are the second leading cause of cancer-related death in children under 15 years old and the third leading cause of cancer-related death in the 15 to 34 year old age group [72]. Approximately 40% of pediatric brain tumors are astrocytic gliomas, of which only 25% are high-grade malignancies [73]. In non-pediatric patients, astrocytomas account for 75 to 90% of primary malignant gliomas, with glioblastoma multiforme representing more than 50% of all malignant gliomas [70].

Increased grade of malignancy of astrocytomas is observed to increase with age [74]. There have been several histopathologic grading strategies utilized for the astrocytoma group. However, within a single histopathologic grade, the prognosis for individual patients is highly variable, since tumors of the same histologic type often progress at different rates and may show distinct responses to the same treatment

therapies [70, 75]. Three tiered grading systems have been demonstrated to be more closely correlated to clinical outcome with the classification scheme of Nelson correlating best with prognosis [76].

The three tiers of histopathologic grading utilized are: in the order of increasing severity of clinical outcome (a) astrocytoma, (b) anaplastic astrocytoma and (c) glioblastoma multiforme. Low-grade astrocytoma is the slowest growing of the tumors with diffuse fibrillary astrocytoma observed most commonly. The histological features are that of increased cellularity and mild to moderate nuclear pleomorphism [70]. The frequency of occurrence of this type of tumor within the brain is similar to the amount of white matter present in each lobe, with the highest incidence found in the frontal lobes [74]. These tumors are diffusely invasive into the surrounding brain with invasion limited to white matter [70]. Pathohistological features of anaplastic astrocytoma demonstrate the presence of more abundant cellularity compared to astrocytoma and one of two nuclear/cellular appearances: (a) nuclear pleomorphism with either scanty cytoplasm and nuclear lobation or (b) abundant eosinophilic cytoplasm of gemistocytes and small uniform nuclei [70]. In addition to these changes there is evidence of increased mitotic activity, the presence of microvascular proliferation and an increase in the bromodeoxyuridine labeling index [70, 77]. Glioblastoma multiforme is the most malignant and most frequently occurring grade of astrocytoma. The major distinguishing factor between anaplastic astrocytoma and glioblastoma multiforme is the presence of tissue necrosis with high cellularity and highly variable pathohistologic morphology [78, 79]. Macroscopic tumor appearance can vary greatly from region to region with the presence of both low- and high-grade features

in different regions of a tumor suggesting that low-grade gliomas can transform to more malignant neoplasms [80]. However, some samples of glioblastoma multiforme are thought to arise directly from malignant transformation of astrocytes [70, 81]. Glioblastoma multiforme tends to infiltrate the brain extensively, giving the appearance of multifocal tumors [70]. However, many of the multifocal sites are found to be histologically connected. True multifocal incidence of glioma occurs in a maximum of 2.5% of astrocytoma cases [82, 83].

Distant metastases are rarely observed in gliomas, but, tumor cells from high grade astrocytomas have been identified in locations of the brain removed from the primary tumor but connected by white matter tracts [84-86]. Glioblastoma cells have been demonstrated to disseminate along white matter tracts [87]. However, as local strategies of tumor control improve, distant metastases have been observed to increase in frequency, leading to the suggestion that more effective systemic therapeutic strategies will be required for tumor control [87].

2.2 Chemotherapy of Malignant Gliomas

Current treatment of malignant gliomas involves surgery, radiotherapy and chemotherapy. Patient survival is influenced by confinement of the tumor within the bony calvarium which limits the ability of the tumor to expand without damage to the host [70]. Most gliomas are not completely resectable due to the infiltrative growth characteristics of malignant gliomas, but treatment by surgical resection followed by radiotherapy and chemotherapy improve survival significantly [88-90]. Patients with high grade gliomas treated by

surgical resection alone have a median survival of 4 to 6 months [91] which reflects the fact that total resection is impossible without causing unacceptable neurological damage to the patient [92]. Following therapeutic intervention (surgical resection, radiotherapy and chemotherapy) in adults, the median survival, on average, of patients with glioblastoma multiforme is approximately one year, with a 20% survival expected after two years [75]. Recently, meta-analysis of 19 random trials, a statistical method that allows results from separate randomized trials to be combined, has demonstrated that adjuvant chemotherapy after primary resection and radiation therapy of malignant gliomas in adults resulted in a small but significant increase in the proportion of surviving patients following resection [93]. The majority of the studies analyzed included a chloroethylnitrosourea. Treatment with radiation alone resulted in a median survival of 9.4 months, whereas treatment with radiation and chemotherapy resulted in a median survival of 12 months [93].

The chloroethylnitrosoureas (CENUs) carmustine (BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea), lomustine (CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) and nimustine (ACNU, 3[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride) appear to be the most active single agents for the treatment of malignant astrocytomas [87]. BCNU is the single most active CENU, with no other drug or drug combination being conclusively more efficacious against malignant astrocytomas [87]. The Brain Tumor Cooperative Group (BTCG [initially called the Brain Tumor Study Group (BTSG)]) initiated a series of clinical studies to assess various postoperative therapies involving nitrosoureas [94-96]. The studies first suggested that BCNU

therapy without radiation was not as effective as radiotherapy alone [94]. Radiotherapy in addition to BCNU treatment did not significantly alter median survival compared to radiotherapy alone but did increase the survival of patients [94, 95]. Further studies by the BTCG suggested that BCNU in addition to radiotherapy modestly improved the median survival of patients with malignant astrocytoma [96] The conclusion of the BTCG studies was that standard treatment of patients with malignant gliomas should include chemotherapy with a nitrosourea [97]. The utilization of nitrosoureas may be complicated by the fact that 40 to 60% of human brain tumors show insensitivity to CENUs and often acquire resistance during therapy [98, 99]. Additionally, nitrosoureas are found, clinically, to produce pulmonary toxicity and delayed cumulative myelosuppression affecting primarily granulocytes and megakaryocytes [100].

Several new adjuvant chemotherapy protocols are currently being analyzed. There is evidence that combination therapy with procarbazine, CCNU and vincristine (PCV) is more efficacious that BCNU alone in patients with anaplastic astrocytoma but not in patients with glioblastoma multiforme [101]. Cisplatin, carboplatin, and cyclophosphamide have recently shown activity against gliomas in non-randomized adjuvant trials [93]. However, as of yet no single agent appears to be as effective as BCNU in randomized trials involving the treatment of malignant gliomas. In addition to the analysis of new chemotherapeutic strategies there has been an increase in the analysis of approaches to increase the delivery of chemotherapeutic agents across the blood-brain barrier to treat high-grade astrocytomas by maximizing delivery of chemotherapy to the tumor (recently reviewed in [87]).

There has been substantial effort on the development of structurally modified CENUs aimed at increasing anti-tumor effect and minimizing toxicity to normal cells [17, 102, 103]. One modification has led to the identification of (2-chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU), an experimental anticancer compound, which is a CENU analog that contains the amino acid amide group N-methylglycinamide, known as sarcosinamide [17]. SarCNU has been demonstrated to be more active, *in vitro*, than the clinically available BCNU in primary glioma cells and glioma cell lines [104]. The same study showed that SarCNU is more cytotoxic to primary human gliomas, *in vitro*, at its theoretical peak plasma concentration (PPC) compared to BCNU at its clinically achievable PPC [104]. Analysis of athymic mice bearing the human glioma cell line U-251 demonstrated that SarCNU was more effective in extending survival than 15 anticancer agents including BCNU [105]. Moreover, SarCNU has been demonstrated to be 10-fold less toxic systemically in mice [17, 105] and 3- to 8-fold less toxic to bone marrow precursor cells, *in vitro*, than BCNU [105, 106]. Additionally, positron emission tomography of patients injected intravenously with tracer ^{11}C-labeled SarCNU and BCNU demonstrated that the ratio of radioactivity present at 60 minutes in a brain tumor as opposed to contralateral brain is substantially larger for SarCNU than BCNU [107]. Analysis of the steady state concentration of SarCNU versus BCNU in a human glioma cell line has revealed that SarCNU levels are 2 fold greater than BCNU, which enters cells via passive diffusion [108, 109]. Furthermore, utilizing sarcosinamide, the carrier group of SarCNU, it has been demonstrated that SarCNU may potentially be taken into cells by an epinephrine sensitive carrier-mediated system. Together, these

results suggest that a carrier mechanism may be involved in the entry of SarCNU into the tumor which may account for the increased effectiveness of SarCNU versus BCNU against gliomas.

3 BIOLOGICAL TRANSPORT

3.1 Diffusion across membranes [110]

Diffusion is defined as the migration of molecules from a region of higher concentration to a lower concentration as a result of their random motion [111]. Diffusion of solutes and organic reagents is the fundamental mode of movement common to biological and non-biological systems. With diffusion, the rate of net transfer of a solute is at all times proportional to the concentration difference that exists across the surface of a membrane through which solute movement takes place. The rate of mass transfer, referred to as velocity of transfer can be expressed as by the symbol υ (mass/unit area of boundary in unit time) and related to the concentration of the diffusing solute, expressed as [S]:

$$\upsilon \propto [S] \qquad (1).$$

In transport kinetics, velocity refers to the change in position of a solute/substrate from one side of the membrane to the other. The velocity of transfer in the opposite direction (υ') can be related to the solute concentration on the other side of the membrane [S']:

$$\upsilon' \propto [S'] \qquad (2).$$

The net rate of transfer (V) at any moment in time is the difference between the two separate rates and is therefore proportional to the difference between the concentrations on either side of the membrane:

$V = (\upsilon - \upsilon')$ (3) or $V \propto ([S] - [S'])$ (4),

with ([S] - [S']) representing the energy difference between the concentrations across the membrane.

At all times, movement of the solute takes place in either direction, therefore the description of diffusion involves the movement of a solute into and out of the cell. The solute molecules must move across the boundary of the membrane which possesses a finite thickness, l, across which there is a progressive change in the concentration gradient. This can be expressed as a difference in the concentration of molecules per unit membrane thickness:

$$\frac{[S] - [S']}{l} \qquad (5).$$

The relationship is now essentially Fick's law, which states that the rate of diffusion of a dissolved substance along a column of fluid is proportional to the concentration gradient [110]:

$$v \propto \frac{[S] - [S']}{l} \qquad (6).$$

In biological terms there is an assumption that there is a uniform gradient across the cell membrane. The concentration of solutes on either side of the membrane can be assumed to be the same (concentration is different from one side to another, depending on the direction of diffusion) if the rate of diffusion within the solvent is faster than through the membrane or if the fluids are well mixed. In the case of the analysis of cellular transport, this requirement is met for the medium and it is assumed that the solute exists in a single well-mixed compartment within the cell.

Many factors influence the velocity of transfer of molecules across a membrane and in any given system they are assumed to be a

constant, the diffusion constant, D. Therefore the net rate of transfer becomes:

$$V = \frac{D([S] - [S'])}{l} \quad (7) \qquad \text{or} \qquad D = \frac{V \cdot l}{[S] - [S']} \quad (8).$$

The diffusion constant, K_D, is equal to D/l, which is essentially a measure of the rate at which molecules of a particular solute penetrate a defined region of membrane. With the definition of K_D, equation (8) transforms to:

$$V = K_D([S]-[S']) \quad (9) \qquad \text{or} \qquad K_D = \frac{V}{[S] - [S']} \quad (10).$$

The K_D constant describes the relationship between the solute, the solvent and the membrane and is, therefore, only partially descriptive of any one of them. All the factors that make up K_D will apply equally in either direction of diffusion which allows K_D to be applied to movement into or out of the cell. Therefore:

$$v = K_D \cdot [S] \quad (11) \qquad \text{and} \qquad v' = K_D \cdot [S'] \quad (12),$$

defining net velocity of movement as:

$$V = (v - v') = K_D([S] - [S']) \quad (13).$$

In an uptake system where substrate/solute transfer occurs entirely through diffusion, a plot of the velocity of substrate movement versus substrate concentration yields a straight line. Equation (13) corresponds to the algebraic equation for a straight line, $y = ax$; the slope, a, represents the K_D of the substrate for that particular cell system.

3.2 Carrier-mediated transport across membranes

Diffusion is by and large the predominant mode of biological transfer of solutes from one side of the membrane to another. However, in many biological tissues, it has been found that the velocity of transfer across a membrane does not increase in proportion to the concentration, but instead approaches an upper limit of velocity at high substrate concentrations. Additionally, metabolically-inert substances have been found to interfer with the transfer of structurally-related substrates without apparent chemical interaction. This type of membrane transfer is referred to as carrier-mediated transport. There are two conditions of equilibrium following carrier-mediated transfer of a substrate: facilitated diffusion has occurred when the ratio of the intracellular versus extracellular concentration of the substrate is approximately equal to unity and concentrative or active transport has occurred when the ratio is significantly greater than unity. Active transport requires the input of energy either through ion gradients or the hydrolysis of ATP, whereas facilitated diffusion requires no such energy input. When a carrier site, assumed to be limited in number on the cell surface, is capable of interacting with any one of many substrates at the same time, a rivalry ensues that is referred to as competition [112]. The identification of a mode of carrier transport for a particular substrate does not preclude the co-existence of diffusion for that substrate.

3.2.1 Theory of substrate adsorption to a membrane carrier

In the presence of a carrier for a particular substrate, the membrane behaves as if it has a limited number of adsorption sites to

which the substrate becomes attached to which are subsequently transferred across the membrane where the molecules become detached. The behavior of carrier adsorption can be related to physical chemistry properties. It has been suggested that the relationship between a gas and an adsorption surface can be expressed in terms of the equation [113]:

$$\theta = \frac{\alpha\mu}{\alpha\mu + \upsilon} \qquad (14),$$

where θ is the fraction of the surface which is covered with gas molecules at equilibrium, μ is the number of gram-molecules of gas striking each unit area of surface per unit time, α is the constant proportion which adheres, and υ is the rate at which the gas would evaporate if the surface were completely covered. This equation can be adapted to describe carrier-mediated transport with the exchange of [S] for μ and $b \cdot Q_{max}$ for υ [112].

Assume that a proportion of substrate molecules in a dilute solution are striking an adsorptive membrane surface and adhering per unit time. Of those that become attached a certain proportion will dissociate per unit time as a function of the stability of the complex. Accordingly, the proportion of attached substrate released will not be directly related to the concentration in solution. Eventually, an equilibrium will be achieved in which the number of molecules being adsorbed will equal the number dissociating per unit time. Assuming this condition is meet, it is possible to relate the total number of molecules present (concentration), the proportion of occupied membrane surface and the number of molecules which are attached and detached per unit time. If m represents the number or mass of molecules in a

solution which strike an unoccupied adsorption surface per unit time, a proportion, r, will adhere per unit time. The number or mass becoming attached is equal to $r \cdot m$. Since mass (m) = concentration x volume, with volume representing the volume in which the molecules striking the surface are contained, then m = [S] x volume, where [S] represents the concentration of the substrate. The number or mass becoming attached, $r \cdot m$, can now be written ($r \cdot$ [S] x volume) or ($\alpha \cdot$ [S]) with α being the association rate constant replacing (r x volume) for a particular substrate in solution. When the adsorptive surface is partly occupied, fewer molecules will become attached than when unoccupied since a smaller surface area of unoccupied membrane is available. The occupied area can be expressed as a fraction , θ or (Q / Q_{max} explained below), of the whole membrane surface where the unoccupied surface becomes defined as the fraction of available surface, $(1 - \theta)$. The number of molecules that are capable of becoming attached per unit time are now a direct proportion of the fraction of unoccupied surface area, $r \cdot m \cdot (1 - \theta)$ or $\alpha \cdot [S] \cdot (1 - \theta)$.

If the mass or quantity of molecules adsorbed to a surface at any instant in time is represented by Q, then the maximum quantity capable of being adsorbed is Q_{max} when the surface is saturated. Per unit time, a fraction, b, of the molecules adsorbed become detached. This fraction can be regarded as the dissociation constant which depends on the stability of the complex. The number of molecules which leave a saturated surface per unit time becomes $b \cdot Qmax$, which is a rate constant for any specified substrate-surface combination. When a fraction of the surface is occupied the number of molecules which leave

per unit time is a fraction of the mass that would leave a saturated surface, $\theta \cdot b \cdot Q_{max}$.

At equilibrium the number of molecules becoming attached equals the number leaving, so that:

$$(1 - \theta)\ \alpha \cdot [S] = \theta \cdot b \cdot Q_{max} \qquad (15),$$

with dimensions on both sides being mass / time. This equation can be rearranged so as to define the proportion of adsorption surface that is occupied, that is , θ, or proportionate saturation:

$$\theta = \frac{\alpha \cdot [S]}{(\alpha \cdot [S]) + (b \cdot Q_{max})} \qquad (16),$$

which rearranges to:

$$\theta = \frac{[S]}{[S] + \dfrac{b \cdot Q_{max}}{\alpha}} \qquad (17).$$

For a specified system the expressions b, Q_{max} and α are the constants as described above. The expression $b \cdot Q_{max} / \alpha$ is therefore a constant and corresponds to the equilibrium constant, or K_S, of physical chemistry with dimensions of mass / volume or concentration, so the equation can now be written:

$$\theta = \frac{[S]}{[S] + K_S} \qquad (18) \qquad \text{or} \qquad \frac{Q}{Q_{max}} = \frac{[S]}{[S] + K_S} \qquad (19).$$

Equations (18) and (19) are in the algebraic form of $y = x / (x + a)$ which describes a rectangular hyperbola where as concentration of the substrate increases, the proportion of adsorption sites occupied increases asymptotically towards saturation where θ approaches 1. With

K_S representing the relationship between association and dissociation, its value determines the proportion of surface occupied at different concentrations of substrate. If the concentration of the substrate equals K_S, then equation (19) becomes:

$$\frac{Q}{Q_{max}} = \frac{K_S}{K_S + K_S} = \frac{1}{2} \qquad (20).$$

The surface is half saturated, therefore a demonstration of the concentration that yields half saturation would define the value of K_S. The relationship between K_S and substrate concentration can also be analyzed with respect to equation (15) where both θ and $(1 - \theta)$ are both equal to $^1/_2$ at equilibrium:

$$^1/_2 \cdot \alpha \cdot [S] = {}^1/_2 \cdot b \cdot Q_{max} \qquad (21),$$

which transforms to:

$$\alpha \cdot [S] = b \cdot Q_{max} \qquad (22),$$

therefore:

$$[S] = \frac{b \quad Q_{max}}{\alpha} = K_S \qquad (23).$$

Hence, no matter what the value of K_S, the proportionate saturation when the substrate concentration equals K_S is $^1/_2$. For any given adsorption surface or carrier Q_{max} is the same if a mole to mole substrate-site relationship is assumed. Therefore, Q_{max} represents the total number of adsorption or binding sites available for a substrate. The values of the association constant, α, and the dissociation constant b, depend on the relationship between the substrate and the carrier. Therefore, a difference in the value of K_S for different adsorptive substrates is due to either a difference in the association or dissociation constants or both.

3.2.2 Kinetic theory of carrier-mediated transport

A carrier may be assumed for the purpose of kinetic description to behave similarly to a number of mobile adsorption sites [112]. Substrate is adsorbed on one side, transported across the membrane and released on the other. In examining the kinetic theory of transport, the simplest kinetic model is utilized as long as experimental data supports the model. We must assume that substrate is present only on one side of the membrane and that over the course of observation the substrate concentration remains effectively zero on the side of the membrane to which the substrate is not applied. We must also assume that the carrier is capable of transporting equal numbers of carrier sites across the membrane in either direction at equal rates. With this assumption, the number of carrier sites will not change during the course of observation.

The theory of mobile adsorption sites for the carrier assumes that the adsorption sites stay at the membrane long enough to come to equilibrium before moving to the opposite side [114]. The proportionate saturation can be expressed the same way for adsorption at equilibrium, but, since the substrate molecules are continuously being removed from exposed surface of the membrane, there is a steady-state. Per unit time, a fraction, β, of the molecules adsorbed to carrier sites become detached at the exposed surface. This fraction can be regarded as the dissociation constant which depends on the stability of the complex and is similar to b for adsorption. Therefore, the number of molecules which leave a saturated carrier system in virtual equilibrium, per unit time, becomes $\beta \cdot Q_{max}$, which is a rate of dissociation constant for any specified substrate-carrier combination. The equilibrium or half-

saturation constant, K_S, can now be replaced with the term, K_m, becoming $\beta \cdot Q_{max} / \alpha$ which is similar to the Michaelis constant in the theory of enzyme kinetics [115]. Equation (18) which describes the proportionate saturation of adsorption sites can now be utilized to describe the proportionate saturation of carrier sites with substrate at the exposed surface:

$$\theta = \frac{[S]}{[S] + K_m} \qquad (24).$$

Molecules that have been translocated and dissociated at the non-exposed side of the membrane are those that have been transported. The mass transported per unit time is referred to as the transport velocity, v. Transport velocity depends on the number of carrier sites available at the exposed surface, the number of molecules that can be adsorbed on each site and the rate of turnover of the sites. If all carrier sites are adsorbed then the maximum quantity, Q_{max}, is available for translocation. Per unit time, a fraction, γ, of the molecules adsorbed to carrier sites become detached at the non-exposed side. The maximal rate of transport is therefore $\gamma \cdot Q_{max}$ which can be abbreviated to V_{max}. The velocity of transport ,v, is therefore a fraction, θ (proportionate saturation), of the maximum velocity of transport, V_{max}:

$$v = \theta \cdot V_{max} \qquad (25).$$

Solving equation (24) with θ from equation (25) yields:

$$v = \frac{V_{max} \cdot [S]}{[S] + K_m} \qquad (26).$$

Equation (26) is the Michaelis-Menten equation [115] which is the simplest model to describe enzyme kinetics and is similar to equation

(19) which describes adsorption of a solute to a membrane surface. Transport data that resembles this equation is referred to as following Michaelis-Menten kinetics and has the same algebraic and graphical properties as the adsorption equation (19) [112]. Equation (26) is in the algebraic form of $y = x / (x + a)$ which describes a rectangular hyperbola where as concentration of the substrate increases, the proportion of transported molecules increases asymptotically towards saturation where v approaches V_{max}. With K_m representing the relationship between association and dissociation (only on the exposed side), its value determines the proportion of carriers occupied at different concentrations of substrate.

When applied to the analysis of transport the value of V_{max}, the maximal rate of drug uptake, is a function of the capacity (Q_{max}) and γ, the proportion of adsorbed molecules which dissociate on the non-exposed side of the membrane of carrier system. The value of Q_{max} is dependent on: (a) the number of carriers on the membrane surface and (b) the molar substrate to carrier site binding ratio. The value of γ is dependent on: (a) the probability that a substrate molecule will dissociate from the carrier site per unit time at the non-exposed side of the membrane and (b) the rate of turnover/mobility of the carrier across the membrane. The Michaelis constant, K_m, represents the substrate concentration at which one-half of the maximal velocity is reached and is related to the affinity of the transport site for the substrate. The K_m in Michaelis-Menten theory reflects three velocity constants similar to K_s of adsorption theory. Therefore, for any given carrier with Q_{max} being the same, if a mole to mole substrate-site relationship is assumed, and the values of the association constant, α,

and the dissociation constant b, depend on the relationship between the substrate and the carrier site, a difference in the value of K_m for different carrier substrates is due to either a difference in α or b or both.

Michaelis-Menten kinetics imply interaction with a limited and constant number of reactive transporter sites. The finding that there is a Michaelis-Menten relationship to the influx of a substrate and its concentration suggests the existence of a carrier mechanism. This relationship also suggests that interaction of the carrier and substrate is rate limiting for cellular entry of most of the substrate, assuming that there is not a large capacity for diffusional entry of the substrate. However, diffusion may contribute significantly to translocation across the membrane, and the velocity of transfer then becomes:

$$v = \underbrace{\frac{V_{max} \cdot [S]}{[S] + K_m}}_{\text{(carrier)}} + \underbrace{K_D \cdot [S]}_{\text{(diffusion)}} \qquad (27).$$

3.3 Inhibition of carrier-mediated transport across membranes

The velocity of substrate transport can be altered by the presence of other compounds, either by interference with the binding of the substrate or the efficiency of transport. Interference with the velocity of transport is termed inhibition. There are two types of transport inhibition, competitive inhibition and non-competitive inhibition. Competitive inhibition occurs when the inhibitor compound interferes with the carrier-site binding of the substrate being examined

for transport. The effect of interference with carrier-site binding is characterized by an increase in K_m. A competitive inhibitor may or may not be transported . Non-competitive inhibition, which includes metabolic inhibition of active transport, involves circumstances where an inhibitor interacts with the carrier to reduce the number of effective carrier-sites or the efficiency of membrane translocation (turnover rate). The effect of this interference is a reduction of V_{max}. Inhibition of transport by inhibitors is distinguished by the alteration of the kinetic constants K_m and V_{max}.

The analysis of competitive inhibition is useful in describing the substrate specificity of a transport system. Analysis of competitive inhibition involves the determination of the equilibrium constant, K_i, which is the concentration of inhibitor that is capable of reducing a substrates velocity of transport by half. The analysis of non-competitive inhibition is useful in determining the metabolic requirements of carrier transport. Metabolic inhibition has been utilized to distinguish between facilitated and active transport when the type of carrier-mediated transport is in question [116]. The determination of the concentration of non-competitive inhibitor that reduces transport by half is not the same as with K_i of competitive inhibition, since the mechanism of action in carrier transport is not the same as occurs with enzyme kinetics [117].

Inhibition of membrane transport of one substrate by an inhibitor does not allow the assumption that the inhibitor and the substrate share the same transporter. Kinetic proof of shared transport of two compounds is deduced from partial tests for identity and should involve as many tests as possible in order to reach a general conclusion [118].

The classic ABC test for shared action at a catalytic site or in the case of membrane transport, a carrier-site, involves two tests [119, 120]. Simply put, each compound should be able to saturate itself and the other candidate for shared transport. For instance, the K_i for substrate A inhibiting substrate B transport should be the equal to the K_m value of the transport of A, and vice versa. Since the measurement of kinetic parameters carry uncertainty a third test has been suggested to be added to the ABC test [118]. The test involves the use of another compound C, presumably transported by the same carrier, which should show the same K_i value as an inhibitor of the uptake of both compound A and B. In the instances where competition is competitive, similar kinetic values for the ABC test strongly suggest that carrier-mediated transport is shared between compound A and B.

3.3.1 Kinetic theory of competitive inhibition of carrier-mediated transport

Competitive inhibition can be viewed in terms of membrane surface adsorption, discussed above, and then modified to apply to carrier-site mediated transport [117]. It is assumed that the substrate (S) and the inhibitor (i) bind to the same site. The probability that the site will be occupied by either depends on the relative concentrations and their relative abilities to remain adsorbed to the sites, in other words, the K_m value of each for the carrier-site. Each compound inhibits the other and possesses its own association , α_S and α_i, and dissociation , b_S and b_i, constants. At equilibrium, each compound occupies a fraction of adsorption sites, θ_S and θ_i, with the fraction of unadsorbed sites being $[1-(\theta_S + \theta_i)]$ which is the fraction of area left for further

association. The rates of association and dissociation for substrate and inhibitor are:

	Association	Dissociation
Substrate	$[1-(\theta_S + \theta_i)] \cdot \alpha_S \cdot [S]$ (28),	$\theta_S \cdot b_S \cdot Q_{maxs}$ (30),
Inhibitor	$[1-(\theta_S + \theta_i)] \cdot \alpha_i \cdot [i]$ (29),	$\theta_i \cdot b_i \cdot Q_{maxi}$ (31),

where [S] and [i] are the concentrations of the substrate and inhibitor and Q_{maxs} and Q_{maxi} are the maximum mass adsorbed of substrate and inhibitor respectively. Equilibrium for both compounds can be expressed as:

Substrate $$[1-(\theta_S + \theta_i)] \cdot \alpha_S \cdot [S] = \theta_S \cdot b_S \cdot Q_{maxs} \quad (32),$$

Inhibitor $$[1-(\theta_S + \theta_i)] \cdot \alpha_i \cdot [i] = \theta_i \cdot b_i \cdot Q_{maxi} \quad (33).$$

Following algebraic manipulation to express equations (32) and (33) in terms of fraction of adsorbed site, one obtains:

Substrate $$\theta_S = \frac{[S]\ (1 - \theta_i)}{[S] + \dfrac{b_S \cdot Q_{maxs}}{\alpha_S}} = \frac{[S]\ (1 - \theta_i)}{[S] + K_S} \quad (34),$$

Inhibitor $$\theta_i = \frac{[i]\ (1 - \theta_S)}{[i] + \dfrac{b_i \cdot Q_{maxi}}{\alpha_i}} = \frac{[i]\ (1 - \theta_S)}{[i] + K_i} \quad (35),$$

where, K_S ($b_S \cdot Q_{maxs} / \alpha_S$) and K_i ($b_i \cdot Q_{maxi} / \alpha_i$) are the equilibrium constants for substrate and inhibitor, respectively. If the expression for θ_i from equation (35) is substituted into equation (34) and algebraic rearrangement and simplification is performed, one obtains:

$$\theta_S = \frac{[S]}{[S] + K_S\,[1 + ([i] / K_i)]} \qquad (36)$$

To describe the rate of transport in the presence of inhibitor, the equilibrium constant for the substrate, K_S, can now be replaced with the term, K_m, becoming $\beta_S \cdot Q_{max_S} / \alpha_S$, similar to the replacement of K_S with K_m for adsorption (see above). Additionally, the velocity of transport of the substrate ,v, is a fraction, θ_S (proportionate saturation), of the maximum velocity of transport, V_{max}, similar to equation (25) we obtain:

$$v = \theta_S \cdot V_{max} \qquad (37).$$

Solving equation (36) with θ_S from equation (37) yields:

$$v = \frac{V_{max} \cdot [S]}{[S] + K_m\,[1 + ([i] / K_i)]} \qquad (38)$$

Equation (38) describes the competitive inhibition of substrate with inhibitor with the "apparent K_m" of the substrate increased by $K_m \cdot ([i] / K_i)$ in the presence of inhibitor. The value of Vmax for the substrate is not altered by pure competitive inhibition. As a fundamental assumption of this derivation, the experimentally determined K_i of the inhibitor should be the same as the value for K_m for transport of the inhibitor, assuming that the inhibitor is transported. If the value of K_i is not representative of K_m of the inhibitor then its value represents the inhibition of total transport by the inhibitor of multiple carrier mechanisms for the substrate. Furthermore, as the concentration of the inhibitor is altered with the concentration of the substrate remaining equal, the velocity of transport of the substrate should behave in a manner predicted by equation (38).

3.4 Experimental determination of passive diffusion

The examination of carrier-mediated transport of a substrate requires that the rate of diffusion be sufficiently low so as not to be detectable or unimportant within the scope of the experimental design. However, diffusion has been demonstrated to contribute significantly to the rate of substrate influx and its rate must be known to define the kinetic parameters of carrier-mediated transport [121]. A plot of the velocity of substrate uptake versus substrate concentration will produce a straight line that passes through the origin and have a slope which represents the diffusion constant, K_D [122]. The measurement of diffusion may be achieved either through physical or chemical means which involve the virtual elimination of carrier-mediated transport. Reduction of temperature and metabolic inhibition are means by which to reduce active transport of substrate but they do not always produce satisfactory inhibition of carrier-mediated transport and reliable estimates of diffusion [122]. However, the saturation of a carrier with high concentrations of the substrate or inhibitor relative to its the K_m or K_i , respectively, will virtually saturate the carrier-mediated component substrate uptake. Any increase in the rate of uptake following saturation can then be attributed to diffusion allowing the estimation of K_D. This technique has been used successfully to estimate diffusion constant for purine nucleobase uptake into human erythrocytes [121].

3.5 Experimental determination of kinetic constants

Michaelis-Menten kinetics applies to situations where one way transfer is occurring. In the analysis of transport, the rate of transport is assumed to be unaffected by passage of time over the time course of initial uptake. However, the ability to measure substrate within a cell is an indication that one way transfer is not the situation but instead net transfer is being measured under initial rate conditions [112]. Accumulation within a cell suggests that substrate movement is occurring in both directions. A compromise in the analysis of carrier-mediated transport is the measurement of influx of substrate, over a short time interval, which is then treated as one way transfer. This measurement is called the initial rate of uptake and its validity as a measure of one way transfer is based on the assumption the concentration of substrate accumulated within a cell over this time period is sufficiently small for outward transfer to be negligible [112]. In fact, uptake curves will generally depart from initial apparent linearity when the internal concentration of free substrate reaches about 20 to 30% of the external substrate concentration [123].

Historically, the analysis of a hyperbolic plot generated by the analysis of membrane transport data with velocity, v, measured for a wide range of substrate concentrations, [S], has involved the rearrangement of the Michaelis-Menten equation, equation (26), to describe a straight line. The plot of v on the ordinate versus [S] on the abscissa reveals a hyperbolic plot which does not readily reveal the kinetic parameters K_m and Vmax. The transformation of transport data to describe a linear function can be performed by plotting 1 / v versus 1 /

[S], plotting v versus v / [S], or plotting [S] / v versus [S] (ordinate versus abscissa) [124-126].

When performing carrier-mediated transport experiments that will be described by linear transformation, it is important to utilize a range of concentrations that extend as far as possible below and above the K_m. If all the concentrations are below the K_m then the estimation of V_{max} becomes unreliable and if all the concentrations are above the K_m then the value of K_m becomes unreliable [122]. Following collection of appropriately controlled data, the conversion of the Michaelis-Menten equation to a double reciprocal plot, 1 / v versus 1 / [S], termed a Lineweaver-Burk plot, is represented by the equation [124]:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (39).$$

The equation is in the form of $y = a \cdot x + b$ with y (ordinate) representing 1 / v and x (abscissa) representing 1 / [S]. The slope of the straight line is equal to K_m / V_{max}, the point at which the line intercepts the ordinate is equal to 1 / V_{max} and the point at which the extrapolated line intercepts the abscissa is equal to -1 / K_m. The plot of v versus v / [S] is referred to as the Eadie-Hofstee plot which is described, following rearrangement of the Michaelis-Menten equation, by the equation [125]:

$$v = V_{max} - \frac{v}{[S]} \cdot K_m \qquad (40).$$

The equation is in the form of $y = b - a \cdot x$ with y (ordinate) representing v and x (abscissa) representing v / [S]. The slope of the straight line is equal to -Km and V_{max} is equal to the point at which the line intercepts the ordinate. The plot of [S] / v versus [S],

following rearrangement of the Michaelis-Menten equation, is described by the equation [126]:

$$\frac{[S]}{v} = \frac{1}{V_{max}} \cdot [S] + \frac{K_m}{V_{max}} \qquad (41).$$

The equation is in the form of $y = a \cdot x + b$ with y (ordinate) representing [S] / v and x (abscissa) representing [S]. The slope of the straight line is equal to $1 / V_{max}$, the point at which the line intercepts the ordinate is equal to K_m / V_{max}, and $-K_m$ is equal to the point at which the extrapolated line intercepts the abscissa.

Kinetic data involves a nonlinear dependence of the observed parameter, velocity, upon the varied parameter, substrate concentration. The traditional methods of transforming nonlinear transport data, discussed above, to linear data perturbs the error distribution of the data. If all velocity data points are equally accurate with respect to standard deviation, then unweighted linear transformation by either the Lineweaver-Burk or the Eadie-Hofstee technique has been demonstrated to significantly increase error associated with increased value along the abscissa [127]. Linear transformation of hyperbolic transport data can be made less error prone by weighted analysis, a statistical procedure that compensates for distorted error distribution [124]. More recently, nonlinear regression has been demonstrated to minimize the differences between experimental and calculated transport data for data in the form [127]:

$$y = f(x, p_1, p_2, \ldots) \qquad (42),$$

where y is a unique function of x and one or more unknown parameters, p_1, p_2, etc. For transport data, the parameters to be determined are Km

and Vmax and the velocity of uptake is monitored versus the substrate concentration.

3.6 Experimental determination of competitive inhibition

In order to determine the value of the equilibrium constant, K_i, it would be of interest to determine if the competition of transport of an inhibitor is completely competitive. When determining inhibition the component of diffusion must first be subtracted from measured velocity values [117]. The method of Dixon is a useful technique that distinguishes between competitive and non-competitive inhibition [128]. With this method, the examination of the intial velocity of uptake of substrate at two concentrations in the presence of increasing amounts of inhibitor is performed by plotting 1 / v versus [i]. By inverting equation (38) and breaking into component parts, one arrives at an equation describing a straight line:

$$\frac{1}{v} = \frac{[S] + K_m}{V_{max} \cdot [S]} + \frac{K_m \cdot [i]}{K_i \cdot V_{max} \cdot [S]} \qquad (43).$$

Since all values except v and [i] are constant, 1 / v is proportional to [i], indicating a linear function.

If the concentration of the inhibitor is changed while two concentrations of substrate $[S]_1$ and $[S]_2$ are utilized, two reciprocal lines are obtained. When plotting 1 / v versus [i] the value on the abscissa where the lines cross is equivalent to $-K_i$. This point of intersection is where the values of 1 / v and [i] will be the same for both equations defining $[S]_1$ and $[S]_2$ uptake in the presence of inhibitor and described by the following equation:

$$[S]_1 (K_i + [i]) = [S]_2 (K_i + [i]) \qquad (44)$$

This is only true when [i] = $-K_i$, since $[S]_1$ and $[S]_2$ are not equal. When [i] = $-K_i$, following insertion into equation (38) and solving , v = V_{max}. Consequently, when the point of intersection of the lines defined by Dixon plot meet with 1 / v being equal or similar to 1 / V_{max} it is assumed that inhibition is solely competitive [128]. With pure non-competitive inhibition 1 / v is not equal to 1 / V_{max} since V_{max} is reduced by this type of inhibition. The point of intersection occurs at the abscissa with [i] = $-K_i$ for non-competitive inhibition [117]. However, if the point of intersection of a Dixon plot occurs above the abscissa but not where 1 / v is equal to 1 / V_{max} then there is a mixture of the two types of inhibition.

3.7 Direct methods utilized to study biological transport

Analysis of transport can be carried out with cells in either monolayer or suspension, assuming the suspension of the cells does not affect the transporters ability to function. The analysis of membrane transport in most instances has been carried out by using tracer amounts of radiolabeled compounds. This technique allows the measurement of accumulated radiolabel, the amount of which is proportional to total substrate accumulation within the cell. Accumulation of substrate can be expressed as amount of intracellular label detected per cell or cell unit or as a ratio of intracellular versus extracellular concentration, usually referred to as cell to medium ratio. In most cases steady-state accumulation of intracellular substrate to concentrations greater than those extracellularly is suggestive of accumulation against a concentration gradient, implying active transport [129]. Steady state accumulation of substrate in equal amounts within the cell versus

extracellularly is generally accepted as a sign of facilitated or passive diffusion [130] whereas levels equal or less than those present extracellularly are thought to indicate passive diffusion [131].

In most simple cases, the analysis of transport involves the addition of substrate to one side of the membrane of the cell, either inside or outside the cell, which is referred to as a zero-trans experiment [132]. Generally, radiolabeled substrate is exposed to the cells from the extracellular surface and the initial rate of uptake is measured. However, demonstration of a carrier mediated transport pathway for a substrate may also be demonstrated by the trans-stimulation or -acceleration [133, 134]. In this technique, cells are preloaded with unlabeled substrate until a steady state equilibrium has been reached which is then followed by replacement of the extracellular medium with lower concentrations of radiolabeled substrate and the initial rate of uptake is measured. The reduction of the extracellular concentration of the substrate disrupts steady state, which causes the carrier to redistribute the substrate to equilibrium. The greater concentration of intracellular substrate accelerates the turnover of the transporter which subsequently causes a temporary acceleration of the initial rate of uptake of radiolabel compared to the zero-trans. This procedure assumes that the influx and efflux of the substrate is mediated by the same carrier and that the rate limiting step of transport is the reorientation of the carrier [135].

3.8 Utilization of cytotoxicity to study biological transport

In the case where a radiolabeled substrate is not available for the assessment of transport potential, cytotoxicity, in the case of cytotoxic agents, may be utilized as an indicator of drug transport. Melphalan is a nitrogen mustard analog that contains a phenylalanine group, the presence of which was postulated to mediate the transport of melphalan via an amino acid carrier [136]. Decreased cytotoxicity of melphalan was observed in the presence of amino acids in the incubation medium. Subsequent analysis has verified the carrier-mediated transport of melphalan by two specific amino acid carriers [137, 138] and demonstrated that altered kinetic parameters, as occurs via apparent K_m in the case of competitive inhibition, can mediate its reduced cytotoxicity [139]. This method is not capable of generating kinetic constants but it is useful in the characterization of the effects of altered transport for cytotoxic compounds either by inhibition of transport by chemical or physical means.

4 TRANSPORT OF AMINO ACIDS

SarCNU contains, as a side chain, an amino acid amide of sarcosine (methylglycine) known as sarcosinamide (N-methylglycinamide) [17]. Transport of sarcosine has been demonstrated to occur through the glycine amino acid transport system known as system Gly [140, 141]. The knowledge that system Gly is able to transport of sarcosine has lead to the proposal that SarCNU uptake may proceed through one of the many amino acid transport systems. Therefore it is necessary to understand the characteristics of the defined amino acid transport systems.

Amino acids exist in one of three forms at physiological pH: (a) dipolar neutral zwitterions (b) tripolar cationic and (c) tripolar anionic. Carrier mediated amino acid transport of all of these forms has been described in a variety of malignant and non-malignant tissues (for reviews [141-143]). The transport of amino acids occurs through many systems that differ with respect to Na^+-dependency and structural requirements related to substrate specificity. The nomenclature of amino acid transport systems was set such that lowercase letters are used to designate Na^+-independent transport systems if a capital letter abbreviation has been selected for a similar Na^+-dependent system [144]. The current terminology of amino acid transport is utilized below.

4.1 Na^+-independent zwitterionic amino acid transport systems

4.1.1 System L

This Na^+-independent system was first described in Ehrlich ascites tumor cells [145, 146]. This system is ubiquitously expressed and has a wide variety of amino acid substrates but has greatest affinity for large hydrophobic, apolar branched chain and aromatic amino acids such as leucine, isoleucine, tyrosine, tryptophan, valine, phenylalanine, methionine and glutamine [141]. A linear correlation over 4 orders of magnitude has been noted between side chain hydrophobicity, as measured by the octanol/water partition coefficient, and the inverse of the apparent affinity of blood-brain barrier system L amino acid substrates [147]. This system is notably responsive to trans-stimulation and is characterized by its ability to exchange substrates across the membrane [141, 148]. By exchange of amino acids, this system allows energy from

a gradient for an amino acid to be redistributed in the form of gradients for a variety of amino acids. This method allows System L to work backward and for several substrates it has been demonstrated that cellular uptake occurs by System A or another Na^+-dependent system and exit occurs through System L [149]. Under physiological conditions net uptake can occur with the absence of exchange [141]. Uptake by this system has been characterized by its ability to transport and be inhibited by 2-aminoendobicyclo(2,2,1)-heptane-2-carboxylic acid (BCH, an isoleucine analog) and 3-aminoendobicyclo(3,2,1)octane-3-carboxylic acid (BCO, a leucine analog) [150, 151]. For BCH, net uptake greatly exceeds the concurrent exit of various intracellular substrate amino acids [152]. System L is partially stereoselective and has a broad pH range in which it can function. Indeed, its specificity can be broadened to include histidine [153] and several amino acids normally taken up by System A [154] when the pH is lowered.

4.1.2 Systems L1 and L2

Systems L1 and L2 are Na^+-independent transport systems first identified in rat hepatocytes that are characterized by their similarity to System L [155]. System L1 shows K_m values in the micromolar range with a low V_{max}, whereas system L2 has K_m values an order of magnitude greater than L1 with a concomitant increase in V_{max}. The expression of the two systems varies with time of culture after initial hepatocyte isolation. System L1 increases with time and system L2 decreases with time in culture. The systems differ with respect to their sensitivity to certain amino acids with L1 being more sensitive to inhibition than L2. However, the possibility remains that the two systems reflect zero-

trans and equilibrium exchange type kinetics for the same transporter, thought to be system L, as a consequence of alterations in intracellular substrate concentration brought about by *in vitro* cell culture [141].

4.1.3 System T

System T is a Na^+-independent transporter first identified in human erythrocytes that transports the aromatic amino acids (benzenoid) tryptophan, tyrosine and phenylalanine [156]. This system is not sensitive to pH changes, moderately stereoselective and is inhibited by BCH [157, 158]. Stereospecificity has been shown with D-tryptophan which is able to competitively inhibit transport but is not a substrate for transport [159]. System T has also been demonstrated in hepatocytes [160, 161].

4.1.4 System asc

System asc was first characterized as a Na^+-independent amino acid transporter in horse erythrocytes [162]. This system is selective for the same group of amino acids as the Na^+-dependent System ASC (discussed below). There is possible heterogeneity in horse erythrocytes leading to the proposal that asc-1 is the original system and asc-2 is a new system with lower affinities compared to asc-1 [163]. System asc has subsequently been identified in the epithelial cells of the human pancreas [164].

4.2 Na^+-independent cationic amino acid transport systems

4.2.1 System y^+

The Na^+-independent system y^+ was originally named system Ly^+ and was described in Ehrlich cells as capable of transporting the cationic amino acids [165]. The system is capable of transporting the cationic amino acids arginine, lysine, ornithine and their homologs and is ubiquitously expressed, except in the liver where its is either not expressed or is present in very small quantities [148, 166]. The system is characterized by its stereoselective preference for L-amino acid isomers, its insensitivity to pH changes, and for accelerative countertransport (trans-stimulation) by intracellular substrates [166, 167]. N^{α}-Methylation of lysine and arginine is not tolerated by the system [166]. The K_i of system y^+ for inhibition of arginine uptake increases as the number of methylene groups separating the α-carbon atom from the guanidino group decreases [166]. System y^+ is believed to accept zwitterionic amino acids as weak substrates in the presence of Na^+ where Na^+ can take the place of the positively charged side chain at y^+ receptor sites [148, 167, 168].

Recently, system y^+ has been characterized as the cell surface receptor for the ecotropic murine retrovirus receptor (EcoR) [169, 170]. Injection of *in vitro* transcribed mRNA of EcoR cDNA in Xenopus laevis oocytes generated the expression of a cell surface protein of a relative molecular mass of 67 kD that possesses 12 to 14 potential membrane-spanning sequences. The topology of the first 8 putative membrane-spanning domains of the EcoR protein have been demonstrated to be homologous with the topology of the arginine and histidine permeases of *Saccharomyces cerevisiae*. Expression of the EcoR protein was found to

be capable of the stereospecific transport of L-arginine, L-lysine, and ornithine independent of the presence of Na^+, and homoserine in the presence of Na^+ without altering the uptake of a panel of zwitterionic and anionic amino acids [169, 170]. Analysis revealed EcoR mRNA in all tissues examined except the liver, consistent with the known distribution of system y^+ [170, 171].

4.3 Other Na^+-independent transport systems

Other less well characterized Na^+-independent systems have been identified in oocytes and pre-implantation blastocysts which are termed system $b^{o,+}$, system b_1^+ and system b_2^+ [142, 143]. System b_1^+ has a preference limited to cationic amino acids whereas system b_2^+ is similar to b_1^+ but has different affinities for certain substrates [143]. System $b^{o,+}$ is the Na^+-independent equivalent to system $B^{o,+}$ (discussed below) [172]. The $b^{o,+}$ system is not sensitive to pH and is capable of interacting weakly with bicyclic amino acids and strongly with the zwitterionic amino acids leucine, isoleucine, and valine as well as the cationic amino acids lysine and arginine. Unlike system $B^{o,+}$, system $b^{o,+}$ is limited to substrates that do not branch at the α or ß positions whereas bulkier groups on the remainder of the side chain appear to enhance interaction [172]. System $b^{o,+}$ appears to transport both zwitterionic and cationic forms of amino acids since a pH shift (6.3 to 8.0) that alters the minor concentrations of cations for leucine and zwitterions for lysine by 50-fold does not affect uptake of these amino acids. The interaction of bicyclic amino acids with system $B^{o,+}$ and not with system $b^{o,+}$ is opposite to the classic interaction of bicyclic

amino acids with the Na^+-independent (system L, and T) and not with the Na^+-dependent (system A, and ASC) transport systems [172].

Two Na^+-independent anionic amino acid transport systems have been characterized [142, 143]. These systems are termed system x^-_G and system x^-_C. System x^-_G preferentially transports glutamate and large anionic analogs whereas aspartate and shorter analogs are transported less well [142]. System x^-_C is similar to system x^-_G, however, this system exclusively functions in an exchange mode and cystine is capable of competing and exchanging with glutamate [142, 143]

4.4 Na^+-dependent zwitterionic amino acid transport systems

4.4.1 System A

The Na^+-dependent system A was discovered as one of two amino acid transport systems present in Ehrlich ascites tumor cells [145]. System A is a target of endocrine regulation and is subject to significant regulation: amino acid starvation induces transport whereas repression occurs in amino acid rich environments and physiological conditions [142, 173]. This system transports a wide scope of amino acids with preference for the short, straight chain amino acids, alanine, glycine and proline, with weaker recognition of branched or cyclic side chain containing amino acids [141, 148]. System A is sensitive to a reduction of pH and tolerates N-methylation, allowing transport of the model substrate of 2-(methylamino)-isobutyrate (MeAIB), which, experimentally, distinguishes System A from System ASC (discussed below) [141, 148]. System A is ubiquitously expressed in both mesenchymal and epithelial cells of mammals but is absent in erythrocytes and reticulocytes [154]. System A utilizes the energy stores inherent in Na^+ ion gradients to

generate steep gradients of various amino acids intracellularly versus extracellularly [142]. The system shows a weak trans-stimulation (acceleration of the initial rate of uptake of an amino acid substrate by preloading with the substrate or analog) and can be made to demonstrate trans-inhibition (reduction of the initial rate of uptake of an amino acid substrate by preloading with the substrate or analog) [174]. System A has also been demonstrated to have variants in certain cell lines [175].

A porcine cDNA has been cloned (SAAT1) which when expressed is capable of mediating the Na^+-dependent uptake of MeAIB which can be inhibited by alanine, serine, cysteine, proline, and glycine but not leucine, glutamate or histidine [176]. The expression of SAAT1 was detected in kidney, liver, spleen and skeletal muscle suggesting a broad tissue distribution. These characteristics indicate that SAAT1 encodes a transport system consistent with system A. The clone encodes a protein of 660 amino acids that is 75% homologous, at the level of amino acid identity, to the human Na^+/glucose cotransporter.(SGLT1) [176].

4.4.2 System ASC

The Na^+-dependent System ASC was discovered in Ehrlich ascites cells when MeAIB failed to completely inhibit alanine uptake [177]. System ASC is ubiquitously expressed and is preferentially stereospecific. It transports L-amino acids with 3 to 5 carbon atoms in a chain such as alanine, serine, cysteine, valine and threonine [118, 141, 154]. A ß-, γ- or δ-hydroxyl or sulfhydryl group intensifies the co-substrate action of Na^+ [142]. System ASC does not recognize N-methylated amino acid derivatives (hence no recognition of MeAIB) and

can function over a broad pH range [141]. However, the system can transport proline, accept 3 to 5 carbon anionic amino acids on protonation (zwitterionic form) and may transport arginine and lysine without Na^+ [142, 148]. System ASC, similar to system A, utilizes the energy stores inherent in Na^+ ion gradients to generate steep gradients of various amino acids intracellularly versus extracellularly [142]. System ASC can generate net uptake of amino acids and demonstrates trans-stimulation [173]. System ASC is not subject to adaptive or endocrine regulation [178]. This system has been shown to show subtle differences with respect to its characteristics in different cell types [154].

System ASC has been demonstrated to show an increased ability to transport anionic amino acids as the pH is brought to values below 6 [148]. A novel designation of system X^-_A was originally given to a Na^+-dependent system capable of transporting cysteinesulfinate (an anionic amino acid analog) in the rat hepatocyte [179, 180]. However, it has subsequently been proposed that identification of system X^-_A occurs through a reversible protonation of system ASC that produces a receptor site capable of accepting anionic amino acids [148]. This is supported by the fact that in human erythrocytes, Ehrlich ascites cells, Chinese hamster ovary cells and pigeon red blood cells there is an increased inhibition of threonine transport (a specific substrate for system ASC) by cysteinesulfinate [180, 181]. When system ASC transports anionic amino acids it has a reduced transport capacity for glutamate and longer amino acid analogs [142].

Recently, two "ASC-like" transporter activities have been cloned from the cDNA of human brain and identified as ASCT1 and SAAT [182,

183]. The cloned transporter activities have relative molecular masses of approximately 56 kD, are highly homologous to each other and are approximately 40 to 45% homologous to the recently cloned mammalian Na^+-glutamate transporters (see below), at the level of amino acid identity [182, 184-186]. Both clones are expressed in all tissues examined with the greatest levels found in the brain, skeletal muscle, and pancreas with lower levels found in the liver, which is an an ASC-rich tissue. Expression of the clones in either Xenopus laevis oocytes (ASCT1) or Hela cells (SAAT) facilitated the Na^+-dependent stereospecific transport of known system ASC substrates. However, expression of the cDNA SAAT was not able to mediate the transport of cysteine. Neither system was able to transport MeAIB, a N-methylated substrate of system A. Clone ASCT1 is similar to system ASC in all respects except that unlike classical system ASC but similar to the Na^+-glutamate transporters it was sensitive to an increase in extracellular K^+. The authors point out that all previous examinations of system ASC have been carried out in cell systems that have multiple partially overlapping transport systems, in terms of substrate specificity [182]. Therefore, the Na^+-dependent substrate specificity of the ASCT1 clone strongly suggests that the cDNA clone encodes the human neutral amino acid transport system ASC [182]. Additionally, clone SAAT may represent one of the multiple system ASC mediated transport activities found in different tissues, since system ASC is usually identified by exclusion of other transport systems and can exhibit significant variability between tissues [183].

4.4.3 System N

The Na^+-dependent system N was originally demonstrated in rat hepatocytes as being capable of transporting the amino acids glutamine, asparagine and histidine [187]. The system shows a preference for the presence of nitrogen in the side chain of amino acids and analogs. The system resembles system A in its high sensitivity to reduction of pH and adaptive regulation but it is not subject to endocrine regulation [187]. System N has been primarily identified in liver but it has also been found in human erythrocytes and a N-like system has been reported in rat skeletal muscle termed N^m [141, 188, 189].

4.4.4 System Gly

The Na^+-dependent system Gly was first characterized as being limited to the substrates glycine and sarcosine in avian erythrocytes [140]. The system has a wide distribution having been identified in human erythrocytes, reticulocytes and hepatocytes [190, 191]. System Gly is similar to system A in its ability to tolerate N-methylation, lack of exchange transport and its high sensitivity to pH [148]. System Gly is also able to tolerate N-ethylation [141]. Also, system Gly is dependent on Na^+ and Cl^- with 2 Na^+ and 1 Cl^- co-transported with glycine [192].

Recently, three groups have cloned the mouse and rat brain high affinity glycine transporters that are absolutely dependent on Na^+ and Cl^- [193-195]. Hybridization analysis revealed that the cloned glycine transporters are expressed primarily in the brain with a faint detection of a potentially cross-hybridizing mRNA species in the liver [193, 194]. The brain specific glycine transporters are capable of transporting

glycine and are inhibited by sarcosine and the ethyl and methyl esters of glycine [193]. The K_m of the brain glycine transporters is in the range of 25 to 125 μM which is significantly greater in affinity than the classical system Gly transporter but pharmacologically similar to the glycine transporter identified in cultured glial cells [196]. The pattern of expression and pharmacological characteristics of the brain-specific glycine transporters suggest that the cloned glycine transporters are distinct from system Gly [196]. These transporters have predicted relative molecular masses of approximately 70 to 72 kD and are approximately 40 to 45% homologous, at the level of amino acid identity, to the members of the GAT1/NET neurotransmitter-transporter superfamily. Therefore, glycine specific transport appears to be mediated through the high affinity brain-specific transporter in the CNS and the low affinity system Gly elsewhere.

4.4.5 System ß

The Na^+ dependent ß system was first identified as being able to transport the ß-amino acids, ß-alanine, taurine and 4-aminobutyric acid, in rat hepatocytes and Ehrlich ascites cells [197]. The transport of ß-amino acids occurs through the interaction of the positive and negative charges of the zwitterion separated by two carbon atoms [198]. The ß system has been identified in cardiac muscle, in the CNS, in blood platelets and in the kidney [198]. The taurine transporter (ß$_T$ system) mouse cDNA has recently been cloned and has been identified to be part of a Na^+- and Cl^--dependent family of related genes known as GAT1/NET that are involved in transport of neurotransmitters (see norepinephrine uptake$_1$ below) [199].

4.5 Other Na^+-dependent transport systems

There are two Na^+-dependent systems characterized for the charged amino acids. System $B^{o,+}$ is expressed primarily in oocytes and early conceptus blastocysts and is a broad scope transport system that is capable of transporting zwitterionic, cationic and bicyclic amino acids [142, 143]. The system is also probably expressed in renal and intestinal brush border cells [143]. System X^-_{AG} is ubiquitously expressed and has an equal preference for the anionic amino acids glutamate, and aspartate as well as other small anionic analogs [142, 143].

4.6 Specific high-affinity L-proline transport

Na^+-dependent high affinity transport of L-proline has been described in rat brain synaptosomes and slices [200, 201]. Recently the high affinity Na^+-dependent proline transporter has been cloned from rat forebrain cDNA and demonstrates approximately 45% sequence identity, at the level of amino acids, to the GAT1/NET neurotransmitter-transporter superfamily [202]. The cloned transporter is stereospecific for L-proline and is inhibited by structurally related compounds including sarcosine, but not by glycine or other neuromodulating amino acids. The expression of the transporter appears to be confined to the CNS since message was not detected in the adrenal gland, rat medullary thyroid carcinoma, or PC12 pheochromocytoma cells. This is consistent with the observation that the high affinity transport of L-proline is unique to nervous tissue [201] whereas low affinity transport of proline has been identified in many tissues [141].

4.7 Specific high affinity transport of glutamate and aspartate (System X^-)

L-Glutamate functions as an excitatory amino acid in the CNS and is removed from the nerve terminal by reuptake into presynaptic nerve terminals and surrounding glia by high affinity transport [203]. High affinity transport of glutamate has been characterized to occur in neurons, glial cells, and epithelial cells of the small intestine and kidney [142, 204, 205]. Currently, there are three distinct systems that have been characterized to be involved in glutamate transport which are: (a) a Na^+-dependent and Cl^--independent (System X^-_{AG}) [206, 207], (b) a Cl^--dependent system [208] and (c) a Na^+- and Cl^--independent system (system x^-_G and system x^-_C) [209]. The high affinity Na^+-dependent transport system has a preference for L-glutamate and D- and L-aspartate [210]. The glial Na^+-dependent transport of glutamate may involve an electrogenic carrier cycle that drives the inward transport of two Na^+ with glutamate and the outward transport of one K^+ and one OH^- or HCO_3^- ion [211].

Recently, three groups have cloned three highly homologous yet distinct Na^+-dependent transporters of L-glutamate and D- and L-aspartate [184-186]. The cloned sequences predict proteins of relative molecular mass of approximately 60 kD and predict a potential for the formation 6 to 10 transmembrane domains. Two of the cloned glutamate/aspartate transporters are brain-specific and characterized as being expressed in glial cells of rats [184, 185]. The other cloned glutamate/aspartate transporter is expressed in the brain (thought to be neuron specific), small intestine, kidney, liver, and the heart of

rabbits [186]. The transporters have been demonstrated to be dependent on external Na^+ and internal K^+, not dependent on Cl^- and able to inhibited by glutamate and aspartate analogs. There is significant sequence homology, at the level of amino acid identity, to the cloned mammalian system ASC transporter and significant regions of homology to the *Escherichia coli* proton/glutamate-aspartate symporter [182, 184-186].

5 TRANSPORT OF CATECHOLAMINES

The characteristics of benzylamine (phenylmethanamine) uptake in Ehrlich ascites tumor cells describes two uptake systems that involve a rapid saturable and a slower, apparently nonsaturable uptake process [212]. The uptake of benzylamine, which bears a N-methylated side chain, was inhibited by epinephrine and norepinephrine. The similarity of benzylamine to sarcosinamide uptake prompted investigation into the possibility that sarcosinamide and SarCNU may share the same catecholamine sensitive carrier [108, 213-215]. The catecholamines, epinephrine, norepinephrine, and dopamine, are involved in nervous system signal transmission. The termination of catecholamine signal transduction involves reuptake and metabolism at the synapse [216-218] by neuronal ($uptake_1$) and extraneuronal ($uptake_2$) transport mechanisms [219-221]. Many synapses in the CNS are surrounded by the processes of astroglial cells (astrocytes) [222]. The perisynaptic location of astrocytes is thought to indicate that they may play a role in transmitter reuptake at the neuronal junction [222-226]. Since uptake into extraneuronal sites is followed by metabolism, the role astroglial cells play in the reuptake of catecholamines has not been well defined

due to insufficient experimental data [218, 222]. However, recently, the uptake$_2$ system has been identified in various primary human gliomas with a non-selective substrate suggesting that extraneuronal uptake$_2$ transport exists in glial cells of the CNS [226, 227]. The transport of catecholamines occurs by the classically defined neuronal uptake$_1$ and extraneuronal uptake$_2$ systems and several as yet undefined mechanisms similar yet distinct from uptake$_1$ and uptake$_2$.

5.1 Physiological concentration of norepinephrine and epinephrine

Recent analysis of the cerebrospinal fluid of normal human subjects has shown the concentrations of norepinephrine to be approximately 1.3 nM (< 64 years of age) to 2.1 nM (≥ 65 years of age) while the concentration of epinephrine was approximately 2.8 nM (all ages) [228]. Furthermore, the axonal concentration of norepinephrine, in rat, demonstrated an increase, upon stimulation, in selected target areas of the locus ceruleus/norepinephrine system from low nM (range from 0.6 to 7.3 nM depending on the anatomical location) at resting state, to a maximum of 2.4 μM [229]. The same study utilized physiologically realistic stimulation frequencies, and demonstrated that the concentration of norepinephrine reaches a maximum of 400 nM at the axonal target area [229]. Plasma concentrations of resting levels of norepinephrine and epinephrine are estimated at 3 nM and 0.3 nM, respectively [230].

5.2 Neuronal ($uptake_1$) uptake of catecholamines.

The importance of the uptake and binding of circulating norepinephrine in tissues represents an important mechanism for inactivation of this catecholamine. The neuronal $uptake_1$ transport of catecholamines functions as an inactivating system that is arranged in series with monoamine oxidase (MAO) and vesicular uptake (described below) which both constitute an intraneuronal "sink" mechanism [217]. About 20 to 30% of translocated norepinephrine translocated into the neuron is deaminated by MAO, while 70 to 80% is stored in vesicles [231, 232]. To date there is no significant evidence to suggest that catechol-O-methyltransferase (COMT) is involved in the intraneuronal metabolism of norepinephrine [217]. However, recent observations suggest that $uptake_1$ can be associated with COMT in certain extraneuronal cell types, including astrocytes, that involves no storage of norepinephrine in vesicles [233-236].

Uptake of norepinephrine was first demonstrated mainly in tissues rich in sympathetic innervation where uptake was greatly reduced after sympathetic denervation [219]. Neuronal transport of catecholamines was identified to be saturable at low perfusion concentrations and localized to nerve endings [220]. The transport of norepinephrine through $uptake_1$ has subsequently been shown to be saturable with the apparent K_m of the system varying from 0.2 to 3 μM [217]. The presence of the neuronal norepinephrine $uptake_1$ transporter has been demonstrated in adrenergic neurons, the adrenal medulla [237] and tumors of neuroectodermal origin such as pheochromocytoma [238], neuroblastoma [239] and medullary thyroidoma [240]. A high-affinity Na^+-dependent uptake of norepinephrine has also been observed in rat astroglial cells [234-236,

241]. The location of astrocytes around synapses and at the periphery of capillaries suggest a role in the regulation of the neural norepinephrine [241].

The neuronal uptake$_1$ transport system is stereochemically selective for the naturally occurring (-)-norepinephrine where the (+)-enantiomer has a five fold reduced affinity for the carrier [220, 242]. However, stereochemical selectivity appears to be lacking in guinea-pig and rabbit uptake$_1$ of norepinephrine [243, 244]. The structural requirements for uptake of catecholamines have been investigated with the use of phenylethylamines with the following conclusions: (a) alkylation of the primary amino group reduces transport (e.g. norepinephrine has a greater affinity than epinephrine and isoprenaline is not a substrate) [245, 246], (b) α-methylation does not alter transport effectiveness (e.g. α-methyltyramine is a good substrate for uptake$_1$) [247], (c) the ß-hydroxyl group is not essential for uptake (e.g. dopamine is as good a substrate as (±)-norepinephrine) [248], (d) the presence of two phenolic hydroxyl groups is not an absolute requirement for uptake (e.g. uptake of phenylephrine (one hydroxyl) and amphetamine (no hydroxyls) has been observed) [249-251] and (e) methylation of phenolic hydroxyl groups reduces transport (e.g. normetanephrine is not a substrate) [252, 253]. Non-phenylethylamines such as serotonin (5-hydroxytryptamine, 5-HT) [254], MPP^+ (1-methyl-4-phenylpyridinium) [255], choline [256] and the adrenergic blocking agents, bretylium [257], guanethidine [258] and bethanidine [259] are also substrates of the uptake$_1$ transporter. The K_{uptake} (V_{max}/K_m) of many substrates of uptake$_1$ varies within a 4 to 5 fold range suggesting a concomitant increase of K_m and V_{max} [217]. Indeed, it has been

demonstrated that V_{max} increases linearly with the K_m for some substrates [259]. The apparent lack of strict specificity of the uptake$_1$ transporter suggests that the common denominator in transported substrates is the presence of an ionizable nitrogen that is not incorporated into an aromatic system [217].

The neuronal uptake$_1$ transporter is extremely temperature dependent [260-262]. Metabolic inhibition, *in vitro*, of uptake$_1$ has been observed with cyanide, anoxia and a combination of iodoacetate (an inhibitor of anaerobic metabolism) with dinitrophenol (an inhibitor of oxidative metabolism) [263-265]. It has been demonstrated that artificial ion gradients can drive uptake$_1$ transport in plasma membrane vesicles, suggesting that ATP generation per se is not required for transport [266]. However, ATP is necessary for the generation of ion gradients in neurons causing neuronal uptake$_1$ transport to be classified as a secondary active transport system [217, 267]. These results are supported by the demonstration that the Na^+/K^+ ATPase inhibitor, oubain, inhibits uptake$_1$ transport [260, 268].

The neuronal uptake$_1$ transporter has been demonstrated to be dependent on external Na^+ with uptake of norepinephrine hardly detectable with low extracellular concentrations of Na^+ [269-273]. Many studies utilizing different Na^+ replacing ions have provided conflicting results as to whether Na^+ effects the K_m and/or the V_{max} of uptake$_1$ transport [217]. However, using the "pure" Na^+ replacement $Tris^+$ ion [273], it was demonstrated that norepinephrine and Na^+ are co-substrates that participate in a two-substrate sequential reaction in which both substrates must bind to the uptake$_1$ carrier in an equal (1:1) ratio [274]. The authors demonstrated, utilizing uptake$_1$ transport analysis

of norepinephrine or Na^+, that the apparent K_m decreased and V_{max} increased when either norepinephrine or Na^+ were increased in extracellular concentration. Additionally, there is indirect evidence that the Na^+ loaded carrier is not transported alone since the neuronal efflux of norepinephrine in Na^+-free medium is inhibited by external Na^+ in a concentration dependent manner [275, 276]. The uptake$_1$ transporter has also been shown to be dependent on external Cl^- which can be effectively replaced by Br^- [277, 278]. Artificial ion gradients can drive uptake$_1$ transport in plasma membrane vesicles providing that there are inwardly directed Na^+ and Cl^- gradients and an outwardly directed K^+ gradient [266]. Replacement of K^+ with Li^+ reduces norepinephrine transport, whereas Rb^+ effectively replaces K^+, suggesting that the uptake$_1$ transporter is also dependent on intracellular K^+ [266].

Cocaine is a non-transported competitive inhibitor of the uptake$_1$ with inhibition of transport of norepinephrine being more potent with the (-)-enantiomer than the (+)-form [220, 279, 280]. Cocaine is a competitive inhibitor with respect to norepinephrine and Na^+ [281]. Another class of non-transported dead-end inhibitors of neuronal norepinephrine uptake$_1$ are the tricyclic antidepressants, desipramine being the most commonly used *in vitro* [217]. The inhibition of norepinephrine uptake$_1$ with tricyclic antidepressants is competitive in nature [282].

The cocaine-sensitive neuronal norepinephrine uptake$_1$ transporter has been cloned [283]. The relative unmodified molecular mass of the transporter was predicted to be 69 KDa and to contain 12 potential transmembrane domains. The mRNA of the transporter was demonstrated to be selectively expressed in the locus ceruleus/norepinephrine system of

the brainstem and in the adrenal gland [283]. The norepinephrine $uptake_1$ transporter (NET) has been shown to belong to a family of neurotransmitter transporters known as the GAT1/NET family [284]. This family of transporters possess significant but dispersed amino acid sequence identities and exhibit similar inferred topographies. So far, this family includes Na^+/Cl^- dependent transporters for γ-aminobutyric acid (rat GAT1 [285], human [286]), serotonin (rat [287]), dopamine (rat [288], human [289]), proline (rat [202]), glycine (rat [193]) and creatine (human [290]). Radiation inactivation analysis of the dopamine $uptake_1$ transporter has suggested that the neuronal $uptake_1$ system functions as a tetramer [291]. Recently, two glutamate/aspartate transporters, which are dependent on Na^+ and K^+ but not Cl^-, have been cloned and were estimated to contain at least 8 potential transmembrane domains [185, 186]. The glutamate/aspartate transporters are postulated to be part of a new family of transporters unrelated to the GAT-1/NET family.

5.3 Extraneuronal ($uptake_2$) uptake of catecholamines.

Iverson first demonstrated that the isolated perfused rat heart contained classical neuronal $uptake_1$ [220] as well as a second extraneuronal $uptake_2$ transport [221] shown to be associated with myocardial cells [292-294]. When discovered, $uptake_2$ was described to differ from $uptake_1$ in that it had a 1000 fold reduction in the affinity for norepinephrine, indicating that $uptake_2$ can play a role at higher extracellular concentrations [221]. The transport of catecholamines through $uptake_2$ has an apparent K_m varying from approximately 20 to 600 μM [218, 263]. Following experiments conducted with inhibition of

catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO), it was concluded that uptake$_2$ can function at all norepinephrine concentrations, however uptake at low concentrations was masked since norepinephrine was rapidly metabolized by COMT and MAO [295]. It has subsequently been shown that the uptake$_2$ is only the first step in an extraneuronal metabolic pathway that involves primarily O-methylation by COMT and to a lesser extent deamination by MAO [218, 296]. At low outside concentrations of catecholamines (up to approximately 1 μM) the extraneuronal metabolizing system functions as an "irreversible, metabolizing site of loss". However, at high outside concentrations of catecholamines, intracellular COMT and MAO become saturated and the metabolizing system functions as a "reversible, accumulating site of loss" [218].

Initial studies in the rat heart indicated that uptake$_2$ sites are located in cardiac muscle cells [297, 298]. Further investigations with high concentrations of exogenously applied catecholamines, *in vitro*, demonstrated accumulation in smooth muscle of blood vessels, splenic capsule, vas deferens, and intestine in a manner similar to uptake$_2$ [299-302]. More recently, uptake$_2$ has been demonstrated to be predominantly located in sympathetically innervated tissues and organs across different mammalian species [218]. In various animals, uptake$_2$ has been identified in (a) myocardial cells (e.g. rat and cat heart [221, 303]), (b) in vascular smooth muscle cells of arteries, arterioles, veins and venules (e.g. human umbilical artery [304]), rat coronary arteries and venules [294]) and (c) in non-vascular smooth muscle of the trachealis, uterus, iris, intestine and spleen [218]. The presence of uptake$_2$ in the CNS is more obscure. 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 [305]. 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 extensively characterized [226].

Uptake$_2$ is a saturable transport system characterized by a higher affinity for epinephrine than norepinephrine, with an even greater affinity for isoprenaline which is not accumulated by uptake$_1$ [246, 263]. The affinity of uptake$_2$ for epinephrine and norepinephrine is significantly lower than uptake$_1$ (higher K_m); however, uptake$_2$ has a larger capacity (higher V_{max}) than uptake$_1$ [263]. The affinity of catecholamines for the uptake$_2$ carrier typically increase in the order: dopamine < norepinephrine < epinephrine < isoprenaline [221, 246, 306]. Investigation of the structure-activity relationship of uptake$_2$ was first investigated by Burgen and Iverson, where uptake was enhanced by N-substitution and O-methylation of catecholamines [306]. Subsequent investigations revealed that uptake$_2$ is sensitive to O-methylated catecholamines [307-309], various corticosteroids (e.g. ß-estradiol and corticosterone, a non-transported inhibitor) [307, 310], ß-haloalkylamines (e.g. phenoxybenzamine) [295, 311] and N-substitution of catecholamines [306]. A more detailed analysis of catecholamine uptake into rat hearts demonstrated that uptake$_2$ is distinct in structure-action relationship from that known for uptake$_1$ as well as the alpha- and beta-adrenoreceptors [312]. The same study revealed that V_{max} increases linearly with the K_m (ID_{50}, reduced affinity) for all substrates tested suggesting that the rate limiting step of uptake$_2$ is

one step in the process of: (a) translocation of the loaded carrier to the inside of the cell (b) release of bound catecholamine and (c) subsequent return of the empty carrier to the outside [312]. Corticosteroids have often been used as specific inhibitors of uptake$_2$, therefore, it is often referred to as "corticosteroid-sensitive" catecholamine transport [218].

Naturally occurring catecholamines are asymmetric at the ß-carbon and uptake$_2$ was originally thought not to exhibit stereochemical specificity for the (-)- and (±)-enatiomers of epinephrine [313] or for the (-)- and (+)-enantiomer of norepinephrine [221]. However, recent evidence in the guinea-pig trachealis, the rat heart and the rabbit aorta suggests that there is a small degree of stereoselectivity for the (-)-isomer [312, 314, 315]. Consistent with Pfeiffer's rule [316], the degree of selectivity was observed to increase in parallel with the affinity of the catecholamine for the carrier (e.g. norepinephrine < epinephrine < isoprenaline) with little to no stereoselectivity observed for norepinephrine [312]. It should be emphasized that the stereoselectivity was small compared with uptake$_1$, with isoprenaline having the greatest difference of 4 to 5 fold [218].

Uptake$_2$ transport is not affected by lack of Na^+, Cl^-, or Ca^{2+} extracellularly [317]. However, uptake$_2$ of catecholamines is dependent on the presence of a K^+ gradient [317, 318]. Depolarization (decrease in negative charge within the cell) of cells by the addition of extracellular K^+ and a variety of other methods has been demonstrated to inhibit uptake$_2$ [218, 319, 320]. The inhibition of uptake$_2$ with extracellular K^+ is due to a decrease in V_{max} of uptake and not to any change in K_m [318]. Uptake$_2$ is not dependent on K^+ per se but on

membrane potential, since hyperpolarization (increase in negative charge within the cell) of guinea-pig trachealis by ß-adrenoreceptor stimulation and with cromakalim, a K^+-channel opening drug, increases the uptake of isoprenaline [321, 322]. Given the uptake$_2$ transport sensitivity to pH and membrane potential and that at physiological pH, greater than 93% of norepinephrine (pK = 8.58, this is the pH at which the protonated and unprotonated species exists at a one to one ratio) is protonated, it has been suggested that the carrier transports the protonated species of catecholamine [320-322]. This is strongly supported, considering that the most potent inhibitors of uptake$_2$, MPP^+ (1-methyl-4-phenylpyridinium) and disprocynium24 (1,1'-diisopropyl-2,4'-cyanine), are permanently positively charged [323-325].

Recently, uptake$_2$ has been identified to occur in the cultured cell lines of human renal carcinoma, Caki-1, and human amnion FL cells [326, 327]. The uptake$_2$ transport of norepinephrine in these cell lines has been demonstrated to be sensitive to inhibitors of the renal transport of organic cations (RTOC), with similar potencies for inhibition of uptake$_2$ and RTOC [326-328]. Renal transport of cations occurs in renal proximal tubules and is responsible for the secretion of organic compounds such as choline, catecholamines, histamine and serotonin [329, 330]. The process of RTOC secretion involves the translocation of organic cations from the blood across the basolateral (also known as peritubular or contraluminal) membrane into the proximal tubule cells and then the translocation across the apical (also known as the luminal) membrane into the urine [331]. The translocation across the basolateral membrane is thought to be an electrogenic, facilitated diffusion driven by the transmembrane electrochemical gradient or an

electroneutral exchange of organic cations [332-336]. Translocation across the apical membrane involves the electroneutral exchange of organic cation for H^+ [332-335, 337]. There are similarities between uptake$_2$ and RTOC; however, various steroids are able to inhibit uptake$_2$ and RTOC with no significant correlation between potencies, suggesting a difference between the two transport systems [328]. Furthermore, decynium22 (1,1'-diethyl-2,2'-cyanine), a potent inhibitor of uptake$_2$ transport, inhibits apical transport but has no effect on basolateral organic cation transport [323, 338]. Although decynium22 is similarly potent in its inhibition of apical cation transport and uptake$_2$, the two systems are clearly distinct since apical transport is driven by H^+ and uptake$_2$ is driven by membrane potential. Interestingly, a polyspecific, membrane potential driven transporter has recently been cloned from a rat kidney cDNA library which possesses characteristics virtually the same as the basolateral RTOC transporter [339]. Northern blot analysis of Caki-1 cells and rat heart detected no hybridization which suggests that the cloned transporter is distinct from extraneuronal uptake$_2$.

The purpose of the extraneuronal uptake$_2$ metabolizing system is unclear. However, it has been suggested that the uptake$_2$ system functions as a "second line of defense" against the overspill of neurotransmitter: it removes from the extracellular space that transmitter which has escaped from neuronal uptake$_1$ [218]. Escape from neuronal uptake$_1$ would not normally occur following neuronal discharge considering that physiologically relevant concentrations at the synaptic cleft have been estimated at a maximum of 400 nM (range of 100 nM to 400 nM), a concentration that would not saturate uptake$_1$ [340]. However, in pathological states such as epilepsy, which involve elevated transmitter

release, uptake$_1$ could become saturated [305]. Furthermore, sodium dependent uptake$_1$ is likely to be impaired during ischemia and/or hypoxia when energy levels fall and sodium ion gradients are disrupted [305]. In fact, recent results have demonstrated, in a human astrocytoma and a human neuroblastoma cell line, that the glutamate/aspartate and uptake$_1$ transporters were impaired by as much as 50% within 5 minutes of initiation of hypoxic conditions [341].

5.4 Non-classical uptake of catecholamines.

There have been several descriptions of catecholamine uptake that do not fully resemble the criteria necessary to be defined as uptake$_1$ and uptake$_2$. One transport system has recently been cloned and identified as the vesicular monoamine transporter(s) (MAT) responsible for the active transport of cytoplasmic catecholamine, accumulated by neuronal uptake$_1$, into intracellular storage vesicles [342]. Several other less well characterized uptake systems have been described that are present at the plasma membrane.

The MAT has been cloned from cDNA libraries of rat pheochromocytoma, and brain as well as a rat basophilic leukemia cell line [342, 343]. The MAT transporters are characterized by: (a) being responsible for the ATP-dependent accumulation of monoamines into secretory vesicles of neurons, enterochromaffin cells, platlets and mast cells (b) broad selectivity for the monoamines serotonin, dopamine, norepinephrine and epinephrine (c) being sensitive to inhibition by reserpine and tetrabenazine (d) not being dependent on Na^+ or inhibited by cocaine and (e) transmembrane H^+-electrochemical dependence [205, 344-346]. There are two MATs for catecholamines, one located in

enterochromaffin cells and capable of mediating resistance to the potent neurotoxin MPP^+ and the other located in the midbrain/brainstem [342, 343]. The brainstem MAT has been localized to dopaminergic, noradrenergic and serotonergic cell populations [342]. The cloned MATs are closely related but show no strong homology to known eucaryotic proteins. However, there is an overall structural homology with the NET family of membrane transporters where the predicted structure contains 12 transmembrane domains and the largest intermembrane loop predicted to reside in the lumenal (MAT) and extracellular (NET) space [342]. However, the transporters function in opposite directions relative to the cytoplasm and have different mechanisms of transport, as described.

Several non-neuronal membrane associated catecholamine transport systems have been identified. There is a cocaine inhibitable, Na^+ and Cl^- dependent uptake of catecholamines in the endothelium of the small vessels of perfused lung which resembles $uptake_1$ but is associated with COMT and MAO [347]. However, a cocaine inhibitable, Na^+ and Cl^- dependent uptake of norepinephrine present in fibroblasts of rabbit dental pulp has recently been identified as $uptake_1$ but also associated with MAO and COMT [348]. This would suggest that both systems are extraneuronally located $uptake_1$. The rat cerebral cortex contains an uptake system for isoprenaline that is associated with COMT and independent of Na^+ but its sensitivity to corticosterone and 3-O-methylisoprenaline is very low thus preventing a definitive classification as $uptake_2$ [349]. The human amnion cell line, FL, has been demonstrated to contain $uptake_2$, however, it also possesses a system of norepinephrine uptake that is resistant to 3-O-methylisoprenaline but can be further inhibited with papaverine by 50%

[327]. 3-O-Methylisoprenaline is classically capable of completely inhibiting uptake$_2$, therefore the papaverine inhibitable portion of norepinephrine is clearly distinct from uptake$_2$. Additionally, there have been two distinct non-neuronal catecholamine uptake systems identified in the rat liver. The first system has been demonstrated to be similar to uptake$_1$ since it is moderately sensitive to Na^+, cocaine, desipramine and ouabain but it is also highly sensitive to the uptake$_2$ inhibitor isoprenaline [350]. Interestingly, both corticosterone and normetanephrine, which are also uptake$_2$ inhibitors, were unable to inhibit norepinephrine uptake in this preparation of hepatocytes. The second system identified in rat hepatocytes is not sensitive to the uptake$_1$ inhibitor desipramine, or the uptake$_2$ inhibitor corticosterone; however, the RTOC inhibitor, cyanine-863, is able to inhibit epinephrine uptake by 62% with no effect on norepinephrine uptake [351]. The identification of these non-neuronal transport systems suggest that there may be alternate routes available for the inactivation of biogenic monoamines in addition to the classically characterized uptake$_1$ and uptake$_2$ catecholamine transporters.

6 TRANSPORT OF ALKYLATING / PLATINATING AGENTS

The structure-activity relationship of physiologically transported substrates and their associated transporter can be exploited to design drugs that can be recognized as a substrate by appropriate transport systems. In the case of alkylating agents, certain instances of drug design have lead to chemotherapeutic compounds that are capable of being transported by a physiologic cellular transporter. However, in other cases involving alkylating agents, the compounds were not specifically

designed to be recognized as transportable substrates but have been subsequently demonstrated to be transported by a physiologic transporter. Certain structural components of these alkylating agents mimic the natural substrate of the transporter which allow for the recognition and carrier-mediated influx of these effective chemotherapeutic agents.

6.1 Transport of nitrogen mustard

The transport of nitrogen mustard (mechlorethamine, HN2), hydrolyzed HN2 (HN2-OH) and its monofunctional analog, dimethyl 2-chloroethylamine (HN1), was first demonstrated in L5178Y murine lymphoblasts [352]. The accumulation of HN2 was observed to act against a concentration gradient of up to 35-fold suggesting that influx of HN2 is an active process. The influx of HN2, was observed to be consistent with Michaelis-Menten kinetics demonstrating saturability, temperature-dependence, competitive inhibition by HN2-OH and intact HN1 and partial inhibition by metabolic poisons ouabain and dinitrophenol. Influx of HN2-OH was also saturable and obeyed Michaelis-Menten kinetics. The affinity of the carrier for the HN2-OH was 3 fold greater than intact HN2 and 20 fold greater than HN1. Additional analysis of HN2 influx into L5178Y lymphoblasts demonstrated that the physiological substrate for the transporter involved in the active accumulation of HN2 was choline [353]. A structural similarity between choline, HN2 and hydrolyzed HN2 was suggested to account for the recognition and transport of HN2 by the choline transporter. The carrier-mediated influx of choline, HN2 and HN2-OH were all mutually competitive with respect to inhibition of specific transport. The transport of choline

also proceeds against a 40 fold concentration gradient and demonstrates a greater affinity (K_m = 250 μM) than HN2 (K_m = 1350 μM) and HN2-OH (K_m = 686 μM). Transport of HN2 through the choline transporter has also been observed in normal and leukemic human lymphoid cells [354] and in Walker 256 rat carcinoma cells [355].

A choline transporter has recently been cloned and is a member of the GAT1/NET superfamily of Na^+- and Cl^--dependent transporters [356]. This transporter is able to mediate the uptake of choline but is not inhibited by the classic choline transport blocker, hemicholinium-3. The carrier-mediated uptake of HN2 by L5178Y lymphoblasts is competitively inhibited by hemicholinium-3 [353] suggesting that HN2 may be transported by a choline transporter subtype characterized by a distinct pharmacological subtype.

Repeated *in vivo* exposure of Ehrlich ascites tumor cells to HN2 has been demonstrated to render them resistant [357]. The resistant cells lines were noted to have an approximate 3 to 4 fold reduction in the accumulation of HN2. Reduced sensitivity to HN2 has, in certain cases, been associated with altered HN2 influx into cells. The first group to analyze HN2 influx kinetics identified a L5178Y cell line that is 10 to 30 fold resistant to HN2 that demonstrated a 2 fold reduction in accumulation of HN2. The reduction was suggested to be a consequence of a slight but significant reduction in affinity and V_{max} compared to wild-type [352]. The reduction in kinetic constants in the HN2-resistant L5178Y cells was considered, at least in part, to account for the resistance of the cell line to HN2. More recently, the transport of choline has been demonstrated to be inhibited by amiloride and dimethylamiloride [358]. Inhibition of the choline transporter with 5-

N,N-dimethylamiloride has been demonstrated to protect Ehrlich ascites tumor cells from the cytotoxic action of HN2 [359]. This same group demonstrated that an Ehrlich Ascites tumor rendered 40 fold resistant to HN2, following step-wise increases in treatment with HN2, possessed an 8 fold reduction in the affinity for choline transport with no alteration in the V_{max} of influx. No cross-resistance to nitrogen mustard analogs chlorambucil or cyclophosphamide was observed which implicates altered transport as the main role in HN2 resistance [359]. Additionally, cytotoxicity induced by HN2 in rat hepatocytes was demonstrated to be reduced, to a significant degree, by the presence of choline, suggesting that the choline carrier is responsible for HN2 uptake in the hepatocytes [360].

6.2 Transport of cyclophosphamide

Cyclophosphamide is a nitrogen mustard prodrug that in order to become cytostatic requires hepatic hydroxylation primarily by three microsomal cytochrome P-450 enzymes (CYP2B6, CYP2C8 and CYP2C9) to produce active metabolites, the primary metabolite being 4-hydroxycyclophosphamide [361, 362]. 4-Hydroxycyclophosphamide equilibrates with ring-opened aldophospamide which undergoes spontaneous decomposition to yield acrolein and the ultimate alkylating species phosphoramide mustard. Influx of intact cyclophosphamide was found to be mediated by a temperature sensitive and partially Na^+-dependent transport process in L5178Y lymphoblasts [129]. Cyclophosphamide influx demonstrated biphasic kinetics with saturability noted at low concentrations and a technically non-saturable component of influx at high concentrations. Transport of cyclophosphamide appears to occur

through facilitated diffusion since accumulation did not proceed past a cell to medium concentration of unity. The influx of cyclophosphamide was not inhibited by several amino acids or by nitrogen mustards, chlorambucil, melphalan, HN2, isophosphamide and activated cyclophosphamide. The entry of activated cyclophosphamide, and ultimately phosphoramide mustard into cells, is thought to occur through the nonpolar 4-hydroxycyclophosphamide, which has been demonstrated to cross the blood-brain barrier and accumulate in the cerebrospinal fluid [363]. As of this moment, the mechanism of influx of 4-hydroxycyclophosphamide into cells has not been addressed but lack of inhibition of cyclophosphamide transport by activated cyclophosphamide suggests that entry is achieved by an independent mechanism.

6.3 Transport of melphalan

Melphalan (l-phenylalanine mustard, L-PAM, Alkeran) is a rationally designed alkylating agent which represents an attempt to link an alkylating agent, mechlorethamine (nitrogen mustard, HN2), to L-phenylalanine [364]. Initial results, utilizing cytotoxicity and analysis of influx as indicators of transport in cultured L1210 murine leukemia cells revealed that melphalan is transported by two separate transport systems [136]. One system of transport was identified to be the Na^+-independent, BCH sensitive system L amino acid transport system. Results obtained in murine LPC-1 plasmacytoma cells and in murine L5178Y lymphoblasts confirmed the involvement of system L and also demonstrated the involvement of the Na^+-dependent system, thought to be system ASC, in the transport of melphalan [137, 138]. The two amino acid transport systems were demonstrated to participate in the energy-dependent

transport of melphalan [137, 138]. Active transport of melphalan at low concentrations is mediated primarily by system ASC but as the concentration of melphalan is increased the participation of system L increases substantially. Efflux of melphalan is thought to occur by passive diffusion [365]. More recently, the uptake of melphalan has been demonstrated to vary inversely with the intracellular levels of calcium where decreased levels lead to enhanced accumulation and increased, levels lead to decreased melphalan accumulation [366].

Decreased melphalan accumulation as a consequence of altered transport has been demonstrated to lead to resistance. The melphalan resistant human rhabdomyosarcoma TE-671 MR xenograft in nude mice has been demonstrated to show a 2 fold reduction in the accumulation of melphalan compared to its wild-type sensitive xenograft [367]. Although TE-671 MR demonstrated partial cross-resistance to the nitrogen mustards cyclophosphamide, ifosfamide and chlorambucil, the level of resistance to melphalan was not due to reported mechanisms of nitrogen mustard resistance but instead altered transport of melphalan appeared to be responsible for resistance. Mutagen pretreatment of Chinese hamster ovary cells lead to the characterization of a mutant cell line defective in system L transport [368]. A clone of the CHO mutant cell line demonstrated decreased transport of system L substrates with an increase in the level of melphalan resistance. Selection of the human breast cancer cell line MCF-7 with step-wise increasing concentrations of melphalan has lead to the isolation of cell line MelR MCF-7 which is 30 fold resistant to melphalan treatment [139]. The resistance was associated with a reduction of accumulation of melphalan associated with alteration of system L. The change in system L in the MelR MCF-7 cell

line was characterized as a slightly increased apparent affinity for melphalan and a 4 fold reduction in the maximal velocity of melphalan transport compared to wild type MCF-7. There was no difference in the ability of either MCF-7 or MelR MCF-7 to efflux intracellular melphalan. However, it has been demonstrated that pre-exposure of MCF-7 cells to a melphalan concentration that produces 90 to 99% cell kill ellicits resistance to further melphalan exposure [369]. This decreased sensitivity was not demonstrated to be associated with altered transport of melphalan into MCF-7 cells.

Although melphalan has been demonstrated to be transported by amino acid transport systems L and ASC *in vitro*, physiologically, the transport of melphalan is inhibited by the presence of high concentrations of large neutral amino acids which compete for the transport of melphalan. The apparent affinity of the blood-brain barrier transporters for amino acids is greater than transporters from other organs [370, 371]. The K_m values of the blood-brain barrier are 10 to 20 fold less (greater affinity) than non-brain tissues and approximate normal concentrations of plasma amino acids [372]. The affinity of the large neutral amino acids for system L in the blood brain barrier display a K_m between 10 μM and 100 μM (K_m = 10 μM for phenylalanine [373]; K_m = 25 μM for leucine [374]) [375]. The rat blood-brain barrier has been shown to transport melphalan via system L with an apparent K_m of 150 μM where saturation of the system occurs at approximately 100 μM, in the presence of amino acids [376]. In the rat, therapeutic concentrations of melphalan range between 0.1 to 9 μM, a concentration that would be insufficient to be transported by system L in the presence of physiologic amino acid concentrations [376]. In

fact, the system L carrier is saturated with plasma amino acids at normal concentrations [375]. However, recently, it has been demonstrated that following fasting and a protein free diet that the levels of large neutral amino acids present in the blood can be decreased by as much as 46% in rats [377]. This decrease in amino acid levels accounted for an increase in the rate of melphalan entry into the brain and a xenotransplanted brain tumor suggesting possible physiological modulation of melphalan uptake. The therapeutic significance of the amino acid lowering diet has not yet been established.

6.4 Transport of D,L-NAM

Many studies have suggested that the placement of a nitrogen mustard group on an amino acid can affect the transport properties of the resulting compound. For instance, the addition of a nitrogen mustard side chain to L-phenylalanine to produce melphalan reduces the affinity of melphalan for system L by greater than 10 fold [375, 376]. Also, the addition of nitrogen mustard on 2-amino-tetrahydro-2-naphthoic acid and subsequent placement of the side chain at one of two positions, 7 or 5, affects the transport affinity by as much as 10 fold [376]. In this regard, D,L-2-Amino-7-bis[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic acid (D,L-NAM) is an analog of melphalan that has been shown to be transported by the large neutral amino acid transporter, system L [378, 379]. D,L-NAM has been demonstrated to be transported by the blood-brain barrier system L transporter with a K_i of 0.2 μM which is a 100 fold greater affinity than for melphalan (K_i = 90 μM) [380]. Influx of D,L-NAM into the brain was found to be saturable, Na^+-independent,

inhibitable with large neutral amino acids, and inhibitable by BCH [381]. The results of kinetic analysis of uptake of D,L-NAM revealed a K_m of 0.19 μM similar to the K_i for the inhibition of leucine uptake by system L. Additionally, the rate of D,L-NAM influx into the brain was found to be 20 fold greater than the rate of melphalan.

6.5 Transport of xylamine

N-2-chloroethyl-N-ethyl-2-methyl-benzylamine (xylamine, a nitrogen mustard) was originally reported to irreversibly inhibit norepinephrine influx into central and peripheral nervous tissues [382, 383]. Analysis of the affect of xylamine on norepinephrine uptake in rat astrocytes via uptake$_1$ indicated that inhibition can occur through a reversible, competitive process and an irreversible process that requires extended periods of exposure to xylamine [384]. The 50% inhibitory concentration (IC_{50}) of xylamine for inhibition of norepinephrine accumulation into astrocytes was found to be approximately 0.8 μM, which is 1000 fold less potent then the classic uptake$_1$ inhibitor, desipramine. Xylamine has also been demonstrated to competitively inhibit the active transport of dopamine into rat striatal synaptosomes with an IC_{50} of 0.2 μM [385]. The binding site of xylamine for the uptake$_1$ transport of norepinephrine is shared with cocaine, as demonstrated in isolated blood vessels [386].

6.6 Transport of chloroethylnitrosoureas

The transport of CENUs has been investigated in order to gain insight as to the mechanism of cellular uptake. The uptake of radiolabeled CCNU and BCNU into murine L5178Y lymphoblasts was demonstrated to be unaffected by temperature and excess extracellular

unlabeled drug. The cell to medium distribution ratio of BCNU did not exceed unity whereas the ratio of CCNU reached a value greater than unity. However, the uptake of CCNU was found to be Na^+-independent and to be unaffected by the presence of metabolic inhibitors. These results are consistent with the accumulation of BCNU and CCNU occurring via passive diffusion [109]. Analysis of cellular uptake of chlorozotocin has been investigated in both murine L5178Y lymphoblasts and murine L1210 leukemia cell lines [387, 388]. Accumulation of radiolabeled chlorozotocin into L5178Y cells never exceeded a cell to medium ratio of unity, is not inhibited by excess unlabeled chlorozotocin, is Na^+-independent and is not affected by metabolic inhibition [387]. Furthermore, accumulation of radiolabeled chlorozotocin in L1210 did not exceed a cell to medium ratio of unity [388]. These results provide evidence that the uptake of the classic CENUs which include BCNU, chlorozotocin and CCNU occurs via passive diffusion.

Recent analysis of SarCNU, a novel CENU, has concentrated on the indirect characterization of its ability to be accumulated in human glioma cell lines [108, 213]. Analysis, via an insensitive colorimetric assay, of the cell to medium ratio levels of SarCNU and BCNU accumulation at steady state demonstrated that SarCNU accumulated to twice the level of BCNU. Furthermore, SarCNU is able to competitively inhibit the uptake of sarcosinamide, the carrier group of SarCNU, which is transported into cells by an epinephrine sensitive carrier-mediated system [108]. Interestingly, steady state accumulation of SarCNU has been characterized to be greater in a SarCNU sensitive versus a resistant human glioma cell line [213]. These results suggest that SarCNU accumulation into human glioma cells may occur through an

epinephrine sensitive carrier which would distinguish SarCNU from other CENUs which enter cells through passive diffusion.

6.7 Transport of platinum based chemotherapy agents

cis-Diamminedichloroplatinum(II) (cisplatin) is one of the most often used anticancer agents involving the treatment of various solid tumors, particularly ovarian, testicular, bladder and head and neck cancers. The most severe side effects of cisplatin treatment are nephrotoxicity, gastrointestinal toxicity, and neurotoxicity which have been reduced by the synthesis of second generation platinum complexes [389]. Platinum complexes consist of chemically stable carrier ligands and labile leaving groups that are usually Cl^- ions, which, in the low Cl^- environment of the cytosol, undergo a series of aquation reactions that generate mono- and diaquo-species that are highly reactive to nucleophilic sites [390]. The major adducts present in cellular DNA, following cisplatin treatment, are intrastrand cross-links. However, formation of DNA-interstrand cross-links, which represent less than 1% of total DNA platination, are considered to play a critical role in the cytotoxicities of platinum analogs [391, 392].

Acquired resistance to cisplatin has been shown to occur by elevations of glutathione, increased levels of metallothioneins, enhanced DNA repair and altered intracellular accumulation (reviewed in [393]). Exploitation of altered intracellular accumulation in resistant cell has been utilized by many groups to investigate the mechanism of entry of cisplatin into cells. Results obtained with non-radiolabeled cisplatin demonstrated lack of saturation of cisplatin influx into cells, which has been taken to suggest passive diffusion [394-396].

However, lack of saturation kinetics is a poor standard for categorizing cisplatin uptake as diffusion [397]. Other groups have suggested that the uptake of cisplatin is carrier-mediated [398], which can be modified by ouabain [399], osmotic strength [400], and cAMP levels [401]. This lack of complete understanding of cisplatin uptake is, in part, due to the lack of a radiolabel with which to perform the appropriate cellular transport experiments.

More recent information, involving the use of radiolabeled platinum compounds, suggests that the accumulation of cisplatin and related platinum derivatives can enter cells through passive diffusion or by an as yet unidentified carrier-mediated process. The analysis of cisplatin transport has often involved the use of a cell line that is exposed to stepwise increasing doses to the drug which subsequently develops resistance compared to the untreated parental cell line. Analysis of the human carcinoma cell line 2008 has demonstrated that accumulation of cisplatin occurs through a process that is inhibited by the metabolic poison, ouabain, which is an inhibitor of the Na^+/K^+ ATPase, but does not demonstrate saturability up to 3 mM cisplatin [116, 402]. This inhibition suggests that the accumulation of cisplatin is either dependent on the electrochemical gradient across membranes or is transported by the Na^+/K^+ ATPase. In addition to ouabain sensitivity, a 2008 cell subline, rendered 3 fold resistant to cisplatin, demonstrated a 50% reduction of accumulation at one hour of exposure associated with an initial accumulation rate that was 66% of the parental cell line [116, 402]. Analysis of another 2008 subline, 2008/C13*5.25, which is 11 fold resistant to cisplatin, demonstrated a 50% reduction of cisplatin accumulation at 10 minutes compared to parental 2008 cells

[401]. The same cell line accumulates approximately 25% and 60% less dichloro(ethylenediamine)platinum(II) (DEP) at 10 and 60 minutes, respectively [403]. Although 2008/C13*5.25 cells accumulate less DEP than 2008 cells, both cell lines were capable of significant accumulation against a concentration gradient of unmodified DEP suggesting the presence of a concentrative mechanism of uptake or an intracellular trap for DEP in both 2008 and 2008/C13*5.25 cell lines. Further investigation revealed that the initial rate of influx of unmodified DEP into 2008/C13*5.25 cells is 3 fold less than the rate for 2008 cells [403]. Therefore, the resistance of 2008/C13*5.25 cells to cisplatin was suggested to be a combination of reduced initial rate of uptake of cisplatin/DEP and a reduced level of platinated DNA-adduct formation. Another study investigated a non-small cell lung cancer cell subline PC-14/CDDP which is 11 fold more resistant to cisplatin than its parental PC-14 cell line [404]. The resistance of PC-14/CDDP cells was associated with a 77% reduction in accumulation of cisplatin compared to PC-14 cells due to an reduced initial influx rate. Ouabain was able to decrease the initial influx rate and accumulation in PC-14 cells by as much as 60% but was unable to affect accumulation in PC-14/CDDP cells. Moreover, lack of ouabain sensitivity in PC-14/CDDP cells was associated with a 40% reduction in the number of extracellular oubain-binding sites compared to the PC-14 parental cell line. The results with PC-14 and PC-14/CDDP cell lines suggest the presence of an ouabain-sensitive uptake process for cisplatin. Investigation of the mechanism of acquired resistance in the human ovarian carcinoma cell line 41M and three cisplatin resistant sublines (41McisR2, R4 and R6) revealed that the level of reduction of cisplatin accumulation corresponded closely

with the resistance factor in each 2, 4 or 6 fold resistant cell subline [405]. Influx of cisplatin was not saturable in 41M cells or resistant sublines at concentrations as high as 500 μM. The reduction in platinum accumulation was in evidence as early as 5 minutes after initiation of cisplatin transport and was not associated with reduced extracellular ouabain-binding sites. Uptake analysis in 41McisR6 cells, 6 fold resistant versus 41M cells, utilizing 5 platinum compound derivatives (JM221, JM274, JM280, JM118 and JM216) demonstrated an ability to overcome the reduced accumulation of cisplatin with a return to platinum accumulation levels similar to the parental 41M cell line. Additionally, there was a positive correlation between platinum accumulation and number of axial carbon atoms present in each compound.

To date, the results obtained for cisplatin accumulation suggest that a significant proportion of uptake occurs through passive diffusion that is somehow effected in certain cisplatin resistant cell lines, as in the case of 41McisR6 cells [405]. However, there is also evidence of modulation of the initial rate of uptake with ouabain, as in 2008 and PC-14 cell lines [403, 404]. The presence of a transport mechanism for cisplatin uptake remains unclear, since there has yet to be a case of cisplatin accumulation that demonstrates saturability. The problem with addressing the presence or absence of an uptake mechanisms arises from the fact that many of the uptake studies involve the analysis of accumulation and not the initial rate of cisplatin uptake. Many studies have performed accumulation analyses after one hour. In the case of 2008 cells, at one hour, only 25% of cisplatin remains in its native form with approximately 40% of total accumulated cisplatin having already reacted with cellular macromolecules [403]. Therefore, the

complete characterization of a transport mechanism for cisplatin requires the analysis of the influx under initial rate conditions coupled with the analysis of cisplatin metabolism, which, to this date, has not been performed.

7 PROPOSED MECHANISMS OF RESISTANCE TO CHLOROETHYLNITROSOUREAS

Innate and acquired resistance to anticancer chemotherapeutic compounds are commonly encountered during the treatment of many cancers. The ability of a tumor cell to combat the affects of anticancer compounds can generally be attributed to altered DNA repair, reduced drug accumulation and increased metabolic inactivation of the drug. With regards to CENUs, there is considerable evidence that MGMT is involved in the repair of CENU modified DNA. However, other DNA repair mechanisms are suggested to be involved in the repair of CENU modified DNA with potential for mediating CENU resistance. There is currently no direct evidence that altered accumulation is able to mediate drug resistance to CENUs. Concerning metabolic inactivation of CENUs, there is a considerable amount of contradictory evidence as to the involvement of metallothionein, glutathione-S-transferase (GST) and glutathione (GSH) in the inactivation of CENUs. The purpose of this section is to review current information regarding the involvement of particular enzymatic systems in innate and acquired resistance to CENUs and there relevance to the clinical antitumor activity of CENUs.

It should be noted that many studies analyzing resistance mechanisms of cytotoxic chemotherapeutic compounds often involve the stepwise exposure of a sensitive wild type cell to increasing drug

concentrations. This selection may result in more than one phenotypic trait involved in resistance to a particular cytotoxic drug, particularly if the stepwise selection occurs over an extended period of time [406]. For instance, elevations of DNA polymerase α and β and topoisomerase II have been identified in a human rhabdomyosarcoma cell line selected for melphalan resistance that is in turn cross-resistant to BCNU [407]. The results suggest that multiple changes in proteins involved in DNA repair have occurred that may contribute to alkylating agent resistance but the significance of each individual pathway is not clear. Therefore it is important to determine if an observed change is directly implicated in or is merely associated with and not essential to acquisition of drug resistance.

7.1 Mechanisms of DNA repair

The relationship between the sensitivity to CENU chemotherapeutic alkylating agents and the efficiency of DNA repair processes has been firmly established [33, 48, 408, 409]. Consistent with the ability of CENUs to generate ISCLs through modification of the O^6 position of guanine, it has been observed that the intermediates in the formation of ISCLs are substrates for the repair protein O^6-methylguanine-DNA methyltransferase (MGMT) [26, 54, 410, 411]. Mammalian MGMT is a M_r 22,000 protein that is capable of catalyzing the stoichiometric and covalent transfer of small alkyl groups, preferably methyl groups, from the O^6 position of guanine to an internal cysteine residue, yielding S-alkylcysteine and unmodified guanine ([412-414] and reviewed in [411]). MGMT is most efficient at removing methyl groups on the O^6 position of guanine but is also able to remove longer alkyl adducts with the rate of

rem~val reduced with increasing length and extent of branching of the alkyl group [415]. The transfer of the alkyl group is referred to as a "suicide" reaction since there is no regeneration of the active cysteine residue thereby inactivating MGMT [416]. A more appropriate description as an incomplete ping pong enzyme mechanism has also been utilized to describe the inactivation of MGMT [411]. The regeneration of MGMT has been reported to occur slowly both *in vitro* and *in vivo* with variation in the time course of enzyme regeneration that is dependent on the origin and type of cell analyzed [417-422]. In fact, the time related attenuation of CENU cytotoxicity has been related to the functional regeneration of MGMT activity [423]. Recently, human MGMT has been characterized as being able to repair O^4-methylthymine *in vitro* [424, 425]. However, the *in vitro* repair of O^4-methylthymine is inefficient compared to O^6-ethylguanine [425] and *in vivo* repair has been shown not to involve MGMT [426].

Tumor cells that possess little or no MGMT activity (termed Mer^- or Mex^-) are much more sensitive to the effects of CENUs with cells that contain a high level of MGMT (termed Mer^+ or Mex^+) being more resistant to the cytotoxic effects *in vitro* and *in vivo* [418, 427-429]. A number of Mer^- cultured tumor cell lines have been found to have increased ISCLs following CENU treatment versus Mer^+ cell lines [33, 430, 431]. The Mer or Mex phenotypes are defined as follows: Mer^+ cells possess an ability to reactivate methylated adenovirus5 [427], while Mex^+ are able to remove O^6-methylguanine residues from DNA following treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [432]. Approximately 20% of cultured human tumor cell lines examined have no detectable MGMT and fall into the Mer^- phenotype [411]. The level of expression of MGMT in

human glioma cell lines has been demonstrated to correlate with the graded methylation of both the promoter and the body of the MGMT gene [433]. Increased methylation of the MGMT gene correlated with decreased levels of MGMT. It appears that the MGMT gene is highly regulated, as indicated by cell type, tissue- and organ-specific variation in MGMT expression [411, 434].

Originally Mer^+ tumor cells were demonstrated to be sensitized *in vitro* to CENUs by treatment with O^6-methylguanine, an inhibitor of MGMT [417, 435, 436]. Pretreatment of a cultured human colon carcinoma cell line with O^6-methylguanine has been demonstrated to increase the number of ISCLs following CENU exposure [437] Subsequently, O^6-benzylguanine has been demonstrated to be a more potent MGMT inhibitor both *in vitro* and *in vivo* than O^6-methylguanine and streptozotocin (a monofunctional alkylating nitrosourea that generates O^6-methylguanine residues in DNA), since benzyl groups are more easily displaced and eliminated than methyl groups [421, 438-440]. The *in vitro* O^6-benzylguanine enhancement of CENU cytotoxicity has been demonstrated to show synergistic efficacy [420]. Mer^- cells which are originally sensitive to CENUs are not sensitive to the CENU cytotoxic enhancement of MGMT inhibition [440]. In fact, the enhancement of CENU cytotoxicity with O^6-benzylguanine is greatest in cell lines with the high MGMT activity both *in vitro* and *in vivo* [441, 442]. Enhancement of the *in vitro* and *in vivo* CENU cytotoxicity has been shown to be more efficient with prolonged depletion of MGMT prior to and following CENU exposure [422, 443, 444]. This requirement for continuous suppression of MGMT activity is consistent with the stability of the O^6,N1-ethanoguanine ISCL precursor of greater than 8 hours [54]. Given the *in vitro* and *in vivo* ability to

increase the cytotoxicity of CENUs with depletion of MGMT, a phase I trial involving the modulation of MGMT activity with streptozotocin with subsequent BCNU treatment was initiated [445]. Although there was significant cellular depletion of MGMT, the maximum tolerated dose of BCNU was also significantly reduced as a consequence of increased toxicity. This suggests that modulation of MGMT in the clinical setting might be possible but will require lower dose ranges of CENUs which may or may not increase therapeutic efficacy of CENU therapy of human cancers.

A significant fraction of human malignant brain tumor cell lines have been reported to be Mer^- [446] which does not appear to be a consequence of gross structural or quantitative abnormalities of the MGMT gene [447]. Normal human brain and tumors generally demonstrate reduced levels of MGMT with regards to other tissue and tumor types [435, 448]. Analysis of surgically resected human brain tumors has demonstrated that a range of 0 to 25% of brain tumors are the Mer^- phenotype [448-451]. In a recent study involving the analysis of gliomas of exclusively astrocytic origin, tumors with no detectable MGMT were found only to be grade IV glioblastoma multiforme [452]. Interestingly, 2 of 3 Mer^- patients responded well to ACNU adjuvant chemotherapy whereas patients with high MGMT values seemed to resist therapy [451]. O^6-benzylguanine pretreatment prior to BCNU therapy has been shown to prolong growth delays and regressions in human brain tumor xenografts, including glioblastoma multiforme [439, 440, 444]. However, a paired comparison of human brain tumors and normal adjacent tissue showed 52% of adjacent normal brain was found to contain no MGMT activity with 60% of paired brain tumor specimens demonstrating

increased levels of MGMT compared to normal brain [450]. This suggests that a number of human brain tumors may have enhanced protection against the cytotoxic effects of CENUs mediated through MGMT. Nevertheless, analysis of the role of MGMT in mono- (MNNG and ENU) and bifunctional (BCNU) nitrosourea resistance in human brain tumor derived cell lines suggests a lack of correlation between MGMT content and sensitivity to both mono- and bifunctional nitrosoureas, especially for ENU [453, 454]. The fact that MGMT appears to provide only limited protection to BCNU supports the proposal of additional resistance mechanisms that persist in the absence of MGMT.

Conversion of Mer^- cell lines to Mer^+ has been brought about by the transfection of human MGMT cDNA into human and rodent cell lines [69, 455-457]. The expression of the human MGMT leads to an increased resistance to the toxicity, mutagenicity and sister chromatid exchange induction by monofunctional and bifunctional nitrosoureas [69, 457, 458]. Interestingly, resistance to the cytotoxicity of ENU, an exclusive ethylating agent, was not altered by expression of MGMT [457, 458]. This is consistent with the observation that, in human brain tumor cell lines, the contribution of MGMT to ENU resistance is very small [453]. The current thinking is that, for ENU, lesions other than O^6-ethylguanine predominate in giving rise to cell killing. Although transfection of human MGMT can mediate increased resistance, the level of resistance to monofunctional nitrosoureas is not linear with regards to MGMT activity [456]. This suggests that, as the dose of nitrosourea increases, lesions other than O^6-alkylguanine become the predominant cytotoxic lesion. Additional transfection studies carried out in nucleotide excision repair deficient and proficient CHO cell lines have

provided further insight into the role of MGMT in CENU resistance [69]. Although the resistance of the excision repair deficient CHO cell line correlated well with levels of transfected MGMT for mono- and bifunctional nitrosoureas, the increased level of MGMT in both cell lines did not reduce the level of sensitivity of the deficient cell line to bifunctional CNU as compared to the proficient cell line [69]. This suggests that MGMT can play an important role in resistance to monofunctional nitrosoureas and partially to bifunctional nitrosoureas but that other lesions can be repaired by DNA excision repair pathways for bifunctional CENUs. In fact, certain Chinese hamster cell lines which lack MGMT activity have been shown to be capable of removing O^6n-butylguanine from DNA [459]. Furthermore, regression analysis of colon cancer cell lines comparing MGMT levels to BCNU sensitivity reveals a linear relationship between MGMT and BCNU but fails to pass through the origin which is suggestive of alternate mechanisms of resistance to CENUs [460]. Together, these results suggest that there are secondary lines of resistance to CENUs which play a varying role in resistance to CENUs depending on the cell origin and type analyzed.

There is evidence that DNA repair functions other than MGMT may play a role in the removal of O^6-alkylguanine adducts [69, 428, 459]. For instance, an eleventh ultraviolet sensitive excision repair-defective complementation group was originally characterized for its sensitivity to CENUs [461, 462]. Comparison between nucleotide excision repair defective and proficient human lymphoblastoid cell lines has provided evidence that nucleotide excision repair participates in the repair of O^6-ethylguanine adducts [426, 463] Furthermore, the transfection of an excision repair defective Chinese hamster ovary cell

line was shown not to decrease the overall level of sensitivity compared to a control transfected repair proficient cell line [457]. The level of participation of nucleotide excision repair in resistance to nitrosoureas remains unclear at this moment, but it is clear that it plays a role in repair of CENU modified DNA. Analysis in human colon cancer cell lines revealed that resistance to CENUs correlated with peak levels of DNA ISCLs but not with MGMT content [464]. Instead, the CENU resistant cell lines that accumulated fewer ISCLs demonstrated an increased ability to repair ISCLs whereas the most sensitive cell appeared unable to repair ISCLs. This is consistent with the observation that the O^6-ethylguanine [465] and the ethylene bridge ISCL generated by CENUs [466] have been reported to be removed independent of MGMT activity which suggests that enhanced ISCL repair may be involved in reduction of cytotoxicity associated with CENUs.

Involvement of 3-methyladenine DNA glycosylase in repair of CENU adducted DNA has been suggested from transfection experiments of *Escherichia coli* glycosylase I (tag) [467], glycosylase II (alkA) [468] and *Saccharomyces cerevisiae* glycosylase (MAG) [469]. Transfection of the *Escherichia coli* tag gene into a chinese hamster cell line resulted in an approximate 50% increase in survival following MNU treatment. However, the level of protection was not proportional to the level of tag protein activity [467]. The survival would appear to be a consequence of the removal of 3-methyladenine since the tag protein has been demonstrated to have no activity on any other DNA methylation product [470]. Under conditions where CCNU treatment of DNA results in 16% 7-alkylguanines it has been demonstrated that purified alkA protein was able to catalyze the release of 7-(2-chloroethyl)guanine, 7-(2-

ethoxyethyl)guanine, 7-(2-hydroxyethyl)guanine and 1,2-bis(7-guanyl)ethane. The release of the 7-alkylguanines was reduced compared to the preferred substrate of 3-methyladenine [468]. The significance of this observation in relation to increased cellular CENU resistance has not currently been investigated. Nevertheless, purified *Saccharomyces cerevisiae* MAG gene product was also shown to be able to release 7-(2-hydroxyethyl)guanine and 7-(2-chloroethyl)guanine from CNU modified DNA. The disruption of the MAG gene increased the sensitivity of *Saccharomyces cerevisiae* cells to CNU [469]. These results suggest that 3-methyladenine DNA glycosylase activity may play a role in repair of CENU modified DNA. However, recent transfection of the human 3-methyladenine DNA glycosylase (alkyl-N-purine DNA glycosylase) into CHO cells did not alter the level of sensitivity to BCNU treatment although expression of repair activity was significantly increased [471]. As of this moment the role of 3-methyladenine DNA glycosylase activity in mediating resistance to CENUs is unclear.

DNA mutator phenotypes have also been associated with increased resistance to the monofunctional alkylating nitrosoureas MNU and ENU [472-474]. A mammalian gene has been cloned that is able to restore UV resistance in post-replication recovery defective Indian muntjac cell line, SVM [475]. This gene has been demonstrated to improve replication of an O-alkylated DNA strand through a hypermutable repair phenotype that partially corrects the repair defect in SVM cells [472]. The spontaneous mutation rate in SVM cells was sufficiently low to rule out involvement of mismatch repair. Interestingly, Hela cells rendered resistant to MNU demonstrate two complementation groups, one which demonstrates an increased spontaneous mutation rate typical of a

mismatch correction repair defect [474]. The relationship of tolerance to MNU and defective mismatch correction has been established in MNU resistant hamster and human cell lines which are defective in binding to guanine:thymidine mispairs and displays a mutator phenotype [473]. The significance of these error prone resistance mechanisms have, to this date, not been investigated with regard to resistance to bifunctional CENUs.

7.2 Drug inactivation

Enzymatic inactivation and detoxification of anticancer drugs may be an important mechanism of tumor cell resistance. BCNU was originally demonstrated to be inactivated by a denitrosation reaction which can be catalyzed by both the cytochrome P-450 monooxygenase system [476] and by GST [477]. Additionally, the cytosolic fraction of mouse liver has been demonstrated to increase a denitrosation reaction of BCNU in the presence of GSH with the formation of 1-(2-chloroethyl)-3-glutathionylethylurea [477]. Most recent studies have concentrated on the involvement of GST in the inactivation of and resistance to CENUs. However, the human metallothionein protein product has also been demonstrated to be able to alter the sensitivity to monofunctional nitrosoureas [478].

It has recently been demonstrated that GSH is able to quench the cross-linking of chloroethylated DNA suggesting a direct ability of GSH to react with and attenuate the cross-link precursor of chloroethylated DNA [479]. Moreover, GSH has also been demonstrated to be able to inactivate the cytotoxic potential of BCNU at physiological pH and

temperature [480]. The significance of these observations in resistance to CENU remains to be elucidated.

The GST supergene family is composed of four subgroups of soluble or cytosolic isoenzymes termed GST class Alpha, Mu, Pi and Theta [481-483]. GSTs are capable of catalyzing the conjugation and inactivation of a variety of electrophilic and genotoxic compounds with the tripeptide, γ-glutamylcysteinylglycine (glutathione, GSH), thereby reducing the carcinogenic, mutagenic and cytotoxic potential of a diverse array of chemical substances [484, 485]. Cytosolic GSTs exist as homo- or heterodimers that can be distinguished by isoelectric focusing properties and enzymatic properties of a particular protein dimer which reflect the subunit composition [486, 487].

The observation that BCNU can be detoxified by a denitrosation reaction catalyzed by glutathione dependent cytosolic GSTs has been extended to demonstrate that class mu subunit 4 isoenzymes are the best catalysts of the *in vitro* denitrosation reaction [488]. Although subunit 4 isoenzymes are the bests catalysts, BCNU is considered to be a poor substrate of the denitrosation reaction demonstrating low maximum reaction velocity and mM K_m values for GSTμ. Studies have identified a rat glioma cell subline (9L-2) 3-4 fold resistant to BCNU which has lower total GST activity but higher activity of the rat GSTμ isoenzymes 3 and 4 subunits [488]. Therefore, the increased resistance of 9L-2 cells may be in part due to the increase in class mu GST content which is supported by the fact that GST inhibition is able to enhance the cytotoxic effect of BCNU in 9L-2 cells. A subsequent study of two human non-small cell lung cancer cell lines (U1810 and U1690)) showed that a human GSTμ 3 subunit was capable of catalyzing the denitrosation

reaction of BCNU and was found only in the resistant cell line, U1810 [489]. Nevertheless, stable transfection of human GSTμ cDNA into the human breast cancer cell line, MCF-7, did not increase the resistance of the transfectants to BCNU even though expression of GSTμ was comparable to 9L-2 cells [490]. Furthermore, lymphocytes isolated from normal human subjects which were positive or negative for the GSTμ 1 (GST M1-1) subunit expression demonstrated no significant differences in the effect of BCNU indicating that the subunit is not an important resistance factor for BCNU [491].

There have also been suggestions that GSTπ may be involved in the resistance to CENUs. In a study utilizing human malignant astrocytoma cell lines, it was demonstrated that GST activity correlated with increased BCNU resistance with GSTπ specifically correlating significantly with the rank order of BCNU resistance [492]. Recently, a human epidermoid cancer cell line (KB) was rendered resistant to buthionine sulfoximine (BSO), a synthetic amino acid that irreversibly inhibits the enzyme γ-glutamylcysteine synthetase, which is critical in GSH biosynthesis [493]. The BSO resistant cell lines showed collateral sensitivity to ACNU and in turn expressed less than 10% of the levels of GSTπ than wild type. Upon transfection of the resistant cell lines with GSTπ cDNA the levels of GSTπ and sensitivity to ACNU were comparable to wild type. These results suggest a possible role for GSTπ in resistance to CENUs.

To date, there are suggestions that cytochrome P-450 and GSTs may be involved in resistance to CENUs, most of which comes from *in vitro* analysis of resistant cell lines. Recent analysis of the murine leukemia cell line L1210 in an *in vivo* model of acquired resistance to

BCNU has demonstrated that cytochrome P-450 may not play a role in BCNU resistance [494]. It was also demonstrated that GSTμ was not detected and expression of GSTπ was reduced in the resistant cell line. This suggests that these GSTs play a minimal role in the resistance of *in vivo* passaged BCNU resistant L1210.

The metallothioneins (MTs) are a group of small (6000-7000 Da), cystein-rich (20 cysteine residues per molecule), cytoplasmic proteins that are ubiquitously expressed and capable of binding to heavy metals [495]. MT appears to be involved with increased drug resistance to certain anti-cancer chemotherapeutic drugs [496]. Involvement of MT in resistance to nitrosoureas has been demonstrated following transfection of human MT-IIA into CHO cells where the resulting cell lines were significantly resistant to MNU and MNNG [478]. However, the protection of MT did not extend to the DNA cross-linking CENUs. Since MT is an extremely nucleophilic species it has been assumed that it can act as a scavenger for highly electrophilic cytotoxic agents. However, the total methylation of DNA by MNU was not altered by the presence of transfected MT, nor did the level of MT parallel the degree of protection to MNU [478]. Indeed, transfection of two alkylating sensitive chinese hamster ovary (CHO) cell lines generated two cell lines one with equal sensitivity to MNU and MNNG and another with significant resistance to MNU and MNNG [497]. Further analysis of CHO mutant cell lines transfected and overexpressing MT showed differential effects with respect to resistance depending on the genetic background [498]. These results suggest that MT is not acting through a scavenger mechanism in the neutralization of alkylating agents but instead acts through an indirect mechanism in the production of resistance to MNU and MNNG.

Recently, a MT CHO transfectant resistant to MNNG but not to BCNU was demonstrated to have marginally less DNA damage caused by MNNG [499]. Furthermore, the resistance to MNNG was accompanied by a more efficient block in DNA synthesis and a longer delay in the G2 phase of the cell cycle following MNNG treatment. These changes were associated with the increased expression of two proteins that may be responsible for resistant phenotype. Together, these results suggest that MT is indirectly responsible for generation of resistance to monofunctional nitrosoureas but not bifunctional CENUs and that the resistance may be a consequence of the ability of MT to modulate the expression of certain proteins able to protect the cell from alkylation.

CHAPTER 2

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

Preface to Chapter 2

In this chapter, the transport of SarCNU was examined directly with radiolabeled SarCNU in human glioma cell line SK-MG-1 cells. The work was initiated in order to confirm previous observations that SarCNU was able to competitively inhibit the uptake of sarcosinamide into SK-MG-1 cells suggesting that SarCNU and sarcosinamide share a common carrier [108]. The same study demonstrated that sarcosinamide was able to competitively inhibit the uptake of epinephrine into SK-MG-1 cells and vice versa. The objectives of Chapter 2 were to determine if uptake of SarCNU is saturable in SK-MG-1 cells and, if so, to determine the kinetic parameters for this process. To this end, the analysis of SarCNU uptake was carried out to determine the effect of temperature and Na^+ on uptake and the saturability/kinetic parameters of SarCNU uptake into SK-MG-1 cells. Additionally, the ability of epinephrine and a variety of amino acids and metabolic inhibitors to inhibit the uptake of SarCNU was examined. Since epinephrine may compete for SarCNU uptake as suggested by results of sarcosinamide uptake analysis, the method of Dixon [128] was utilized in order to determine whether inhibition with epinephrine is competitive or

noncompetitive. All experiments in Chapter 2 were performed by the candidate excluding the analysis of intracellular metabolism by thin layer chromatography, which was performed by Areti Malapetsa.

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 linear to 4 seconds at 37°C. Equilibrium was reached after 1 minute at 22 and 37°C with accumulation slightly above unity. SarCNU was not significantly metabolized in the cells, following a 60 minute incubation at 37°C, as shown by thin layer chromatography. At 37°C, uptake of [^{3}H]SarCNU was found to be saturable, sodium independent, and energy independent. Previous work has 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 transport of epinephrine physiologically. 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 of 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 inhibits the uptake of SarCNU competitively, with a K_i= 163 ± 15 μM, a value similar to the K_m value of epinephrine influx in SK-MG-1. Additionally, following appropriate corrections for nonfacilitated diffusion to 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= 2.39 ± 0.37 mM and V_{max}= 236 ± 53 pmol/µl intracellular water/second. 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 is mediated by both nonfacilitated diffusion and by facilitated diffusion via the catecholamine uptake 2 carrier system. SarCNU is the first chloroethylnitrosourea that has been demonstrated to have a carrier mediated uptake. Moreover, this carrier mediated uptake may play a rcle in the increased cytotoxicity of SarCNU against gliomas as compared to BCNU, which enters cells primarily by passive diffusion.

INTRODUCTION

SarCNU, an experimental anticancer compound, is a chloroethylnitrosourea (CENU) analogue that contains the amino acid amide group N-methylglycinamide, known as sarcosinamide [17]. 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* [104], and in human glioma cells intracerebrally implanted into nude mice [105]. In addition, SarCNU is less toxic than BCNU in mice [17] and less myelotoxic in the *in vitro* CFU-C assay with normal human bone marrow [106]. Further, SarCNU is more cytotoxic *in vitro* at its theoretical PPC of 68 μM compared to BCNU at its clinically achievable PPC of 9 μM [104]. The presence of the sarcosinamide carrier moiety in SarCNU suggests that transport into cells may be altered as compared to BCNU and other clinically available CENUs, which enter cells via passive diffusion [109].

Previously, the hypothesis that SarCNU may not enter cells purely by passive diffusion was tested indirectly by utilizing a modified version of cytotoxicity as an indicator of transport of an antitumor agent [136, 500]. 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 not BCNU, suggesting that SarCNU may not enter cells purely by passive diffusion [500]. Additionally, kinetic analysis of sarcosinamide influx was used as an indirect measure of SarCNU uptake. Sarcosinamide was utilized as it was expected to be the native

substrate for the transport of SarCNU and was thought to be the more appropriate for the 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. 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 both 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 [108].

In order 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 a concentration in the range of the theoretical PPC of SarCNU in SK-MG-1 cells. The method of Domin et al. [121] was used to estimate the diffusional component of SarCNU accumulation by utilizing excess amount of the co-permeant, epinephrine. The kinetic parameters (K_m and V_{max}) of SarCNU were defined, following correction of SarCNU accumulation for diffusion. Additionally, the method of Dixon [128] 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, Japan. It was dissolved in 0.001 M citrate buffer, pH 4.0, aliquoted, and stored at -20°C.

Materials. (±) Epinephrine HCl, and all unlabeled amino acids were obtained from Sigma Chemical Co. (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. [17]. Briefly, [^{3}H]sarcosinamide was prepared by use of a catalyzed exchange reaction with high specific activity tritiated water and the crude preparation 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 (2-chloroethyl)-3-[^{3}H]sarcosinamide-1-nitrosourea which was purified by HPLC. The radiochemical purity was greater than 99%, using thin layer chromatography on No. 13179 silica gel plates (Kodak), in a solvent system of butan-1-ol:4.0 M sodium acetate pH 4.0:water (4:2:1) with an R_f value of 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, Quebec, Canada. Dextrose and NaCl were obtained from Fisher

Laboratories. Trizma 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). 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°C. 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 x g for 5 minutes, washed twice in PAG and resuspended in PAG to either $4x10^6$ or $2x10^6$ cells/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 of 6.84 µCi/µmol), was assayed on confluent monolayers of SK-MG-1 cells ($1x10^6$ cells/35 mm plate) at 22°C as previously described [108, 501]. In the second method, at 0, 22, and 37°C, [^{3}H]SarCNU, at a 50 µM concentration (specific activity of 6.84 µCi/µmol), was used to assay transport in SK-MG-1 cells, in suspension, with a modified version of "oil-stop" methodology [108, 136, 502]. The papaverine stop methodology, utilized for purine nucleobase uptake analysis, which allows for earlier time

points, was not satisfactory since the method did not prevent diffusion of SarCNU [121]. 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 ($4x10^6$ cells/ml) and [^{3}H]SarCNU solution (hereafter called permeant) were preincubated for 15 minutes at 0, 22, and 37°C. Transport was initiated by rapid addition of 200 µl of cell suspension to 200 µl of permeant (final SarCNU concentration of 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 seconds, was performed on cell suspensions ($2x10^6$ cells/ml) preincubated for 15 minutes at either 22 or 37°C to which an aliquot of [^{3}H]SarCNU was added (final [^{3}H]SarCNU concentration of 50 µM). At indicated time points, assays were terminated by separating the cells from the media by centrifugation in a Brinkman-Eppendorf microcentrifuge (14,000 x g) for 30 seconds. A sample of media was removed for liquid scintillation counting, followed by aspiration of media and silicone oil with subsequent inversion of the microcentrifuge tubes. Contaminant media 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 minutes. Cell debris was removed by centrifugation (14,000 x g) of the lysate for 3 minutes and supernatant placed in 10 ml of Ecolite (+) scintillation fluid (ICN) and counted in a Packard Tri-carb model 1500 liquid scintillation analyzer. The intracellular water space (ICW) was determined, after mixture of cell suspension and unlabeled permeant with tritiated water and [carboxyl-^{14}C]inulin, by centrifugation of cells through silicone oil, as previously described [108, 503]. Analysis of membrane associated

SarCNU radioactivity ("zero-time") was performed as above with immediate centrifugation following addition of cell suspension to [^{3}H]SarCNU suspension. The "zero-time" point requires 0.5 seconds (as determined by metronome) and each time point was actually the time stated plus the 0.5 seconds (not including the time required for the cells to traverse the oil). [Carboxyl-^{14}C]inulin contamination, representing extracellular water space within a cell pellet, contributed to 0.33 ± 0.02 µl per 1 µl intracellular water space at all temperatures, drug concentrations, and reagents tested. This media 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 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 performed in quadruplicate. Cell viability was determined to be greater than 95%, by trypan blue exclusion, in all experiments.

Kinetic analysis and inhibition of uptake was performed at 37°C, as described above, except various concentrations of osmotically adjusted [^{3}H] SarCNU (final specific activity from 6.84 µCi/µmol to 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 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 terminated at the indicated time, as described. Media contamination of cell pellets was subtracted from every experimental determination, as

described. The "zero-time" point was performed separately for every different drug concentration and inhibitor agent used. Initial transport velocity was measured at 2 seconds (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 previously utilized [108, 129]. Briefly, cells were pretreated for 15 minutes at 37°C, prior to examining the uptake of [^{3}H]SarCNU, at a concentration of 50 µM, at "zero-time" and 2 seconds, as described. The ICW was determined, individually, for each different pretreatment, as above.

Measurement of trans-stimulation of [^{3}H]SarCNU uptake. See Appendix 1.

Metabolism of [^{3}H]SarCNU. Samples obtained from transport assays were utilized in these experiments. Cell suspensions ($2x10^6$ cells/ml) were incubated with [^{3}H]SarCNU, at a 50 µM concentration (specific activity 6.84 µCi/µmol), for 60 minutes at 37°C and 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°C 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 [127] 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 analysis 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 Technique. Monolayer accumulation studies were initiated to study the uptake of [^{3}H]SarCNU at 37°C and 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 were not able to increase the cell adherence to the substratum. However, the problem of cell adherence was not as apparent at 22°C, which made it possible to compare the monolayer technique to the suspension technique that has been modified to perform accumulation studies at 37°C. Figure 1 shows that at 22°C, in SK-MG-1 cells, both techniques proved to be reproducible and demonstrated that SarCNU accumulation is linear to 6 seconds and begins to plateau at 60 seconds. 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 $\mu l/10^6$ cells. This value, obtained for monolayers, is only an estimate, since, cells on a monolayer are elongated whereas cells in suspension are spherical. However, the estimated cell to medium ratio in monolayers, at 10 minutes, was similar to that determined in suspension (1.46 ± 0.08 versus 1.23 ± 0.06, respectively). Following a 20 second incubation in SK-MG-1 cells, at 22°C, with drug added directly to cell suspension or 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, since, with this method, time points as early

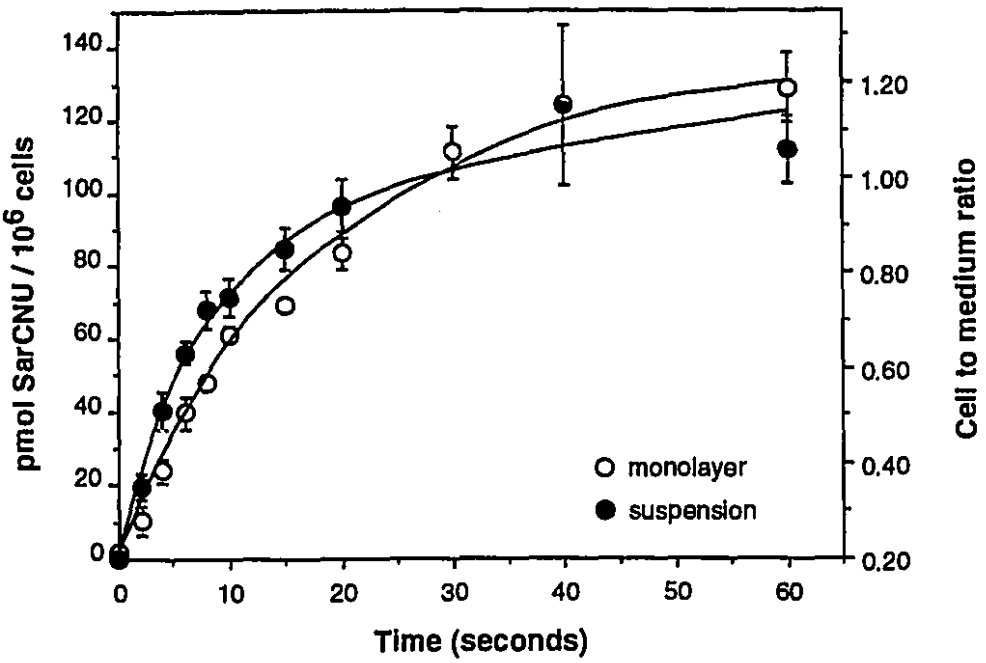


Figure 2.1. Comparison of the time course of the uptake of [^{3}H]SarCNU, at a 50 μM concentration, by SK-MG-1 cells in suspension and in monolayers at 22°C. The uptake, in suspension, is expressed as cell to medium distribution ratio, as described in "Materials and Methods." The uptake, in monolayer, is expressed as pmol SarCNU/10^6 cells. Points, mean of at least 4 separate experiments with each time interval performed in quadruplicate; bars, SE.

as 2 seconds can be obtained in contrast to 15 seconds for direct addition of drug to the cell suspension.

Time Course of Uptake of [^{3}H]SarCNU at Different Temperatures. A time course, performed at 0, 22, and 37°C, of uptake of [^{3}H]SarCNU, at a 50 μM concentration, in SK-MG-1 cells in suspension, is shown in Figure 2. At 37°C, uptake of SarCNU was linear to 4 seconds and begins to reach equilibrium after 1 minute. Additionally, analysis of the initial rate of accumulation at SarCNU concentrations of as great as 50 mM were found to be linear to 4 seconds (results not shown). The differences, at various temperatures, between the accumulation of SarCNU at 2 seconds were found to be statistically significant. The values were 0.20 ± 0.08, 0.35 ± 0.03, and 0.59 ± 0.05 at 0, 22, and 37°C, respectively ($p < 0.025$ for 37 versus 22 and 22 versus 0°C, plus $p < 0.005$ for 37 versus 0°C). All subsequent kinetic experiments were carried out at 2 seconds to approach the initial rate of uptake conditions, and in order to minimize the effects of efflux as the cell to medium ratio, minus "zero-time", was below 0.3 at this point [123].

Steady State Accumulation of [^{3}H]SarCNU. The cell to medium ratio in SK-MG-1 cells, at 22 and 37°C, after a 30 minute incubation with 50 μM [^{3}H]SarCNU was 1.08 ± 0.05 and 1.26 ± 0.07, respectively. These results, utilizing 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 [108].

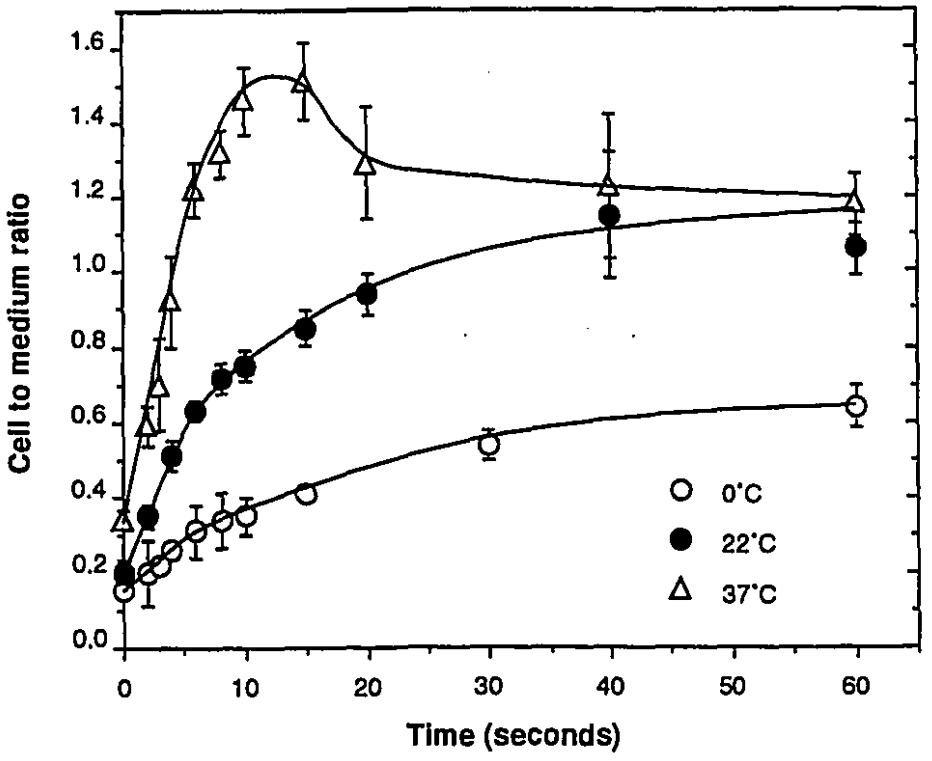


Figure 2.2. Time course of uptake of [^{3}H]SarCNU, at a 50 μM concentration, by SK-MG-1 cells in suspension at 0, 22, and 37°C. The uptake, in suspension, is expressed as cell to medium distribution ratio, as described in "Materials and Methods." The "zero-time" cell to medium ratios were 0.150 ± 0.002, 0.20 ± 0.03, and 0.34 ± 0.02 for 0, 22, and 37°C, respectively. Points, mean of at least 4 separate experiments with each time interval performed in quadruplicate; bars, SE.

Trans-stimulation of [^{3}H]SarCNU Uptake in SK-MG-1. See Appendix 1.

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 [108]. 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 (see Appendix 2). 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), and glycine (Gly 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 for 10 mM SarCNU, $p < 0.05$, and 3.75 ± 0.25 pmol/μl ICW/sec for 10 mM epinephrine, $p < 0.005$). These results suggest that SarCNU, and epinephrine are carried by a common transport system, as previously demonstrated [108].

Effect of Sodium on Uptake of [^{3}H]SarCNU in SK-MG-1. The initial rate of accumulation, at 37°C, of [^{3}H]SarCNU, at a 50 μM concentration, was analyzed for SK-MG-1 cells with either normal PAG medium containing 140 mM NaCl (151.2 mEq/L of Na^+) or PAG medium containing 140 mM Tris pH 7.4 replacing NaCl (11.2 mEq/L of Na^+). In 3

paired experiments, at 37°C, the cell to medium ratio at 2 seconds 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 (see Appendix 3). Pretreatment of SK-MG-1 cells, with the metabolic poisons, did not affect viability with greater than 95% of cells excluding trypan blue after a 60 minute incubation at 37°C. In 4 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 SarCNU concentration is increased from 50 μM to 7.5 mM the cell to medium ratio, at 2 seconds, decreases from 0.63 to 0.52. Thereafter, there was no further decrease in SarCNU distribution up to a 50 mM concentration (results not shown). The inability to reduce the cell to medium ratio of SarCNU below approximately 0.52 at concentrations of greater than 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

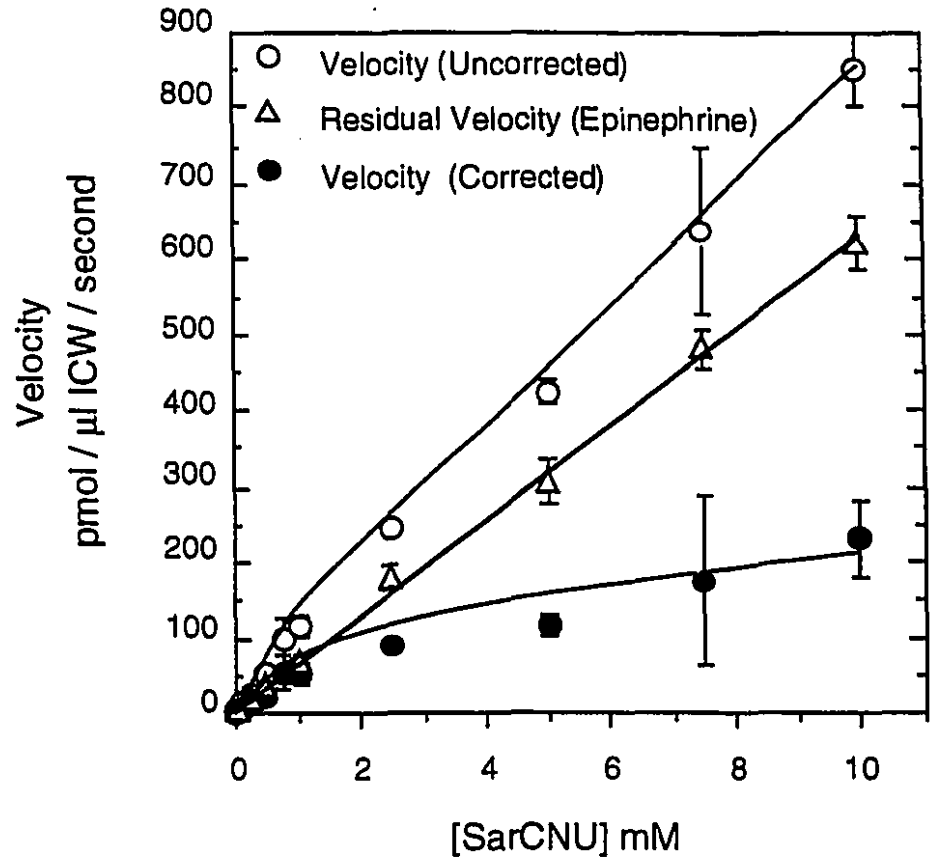


Figure 2.3. Uptake of [^{3}H]SarCNU as a function of concentration. The 2 second uptakes of SarCNU from 50 µM to 10 mM in the presence (residual velocity) and absence (velocity) of 10 mM epinephrine were measured in suspension at 37°C, as described in "Materials and Methods." The corrected curve represents the velocity of 50 µM to 10 mM SarCNU minus the residual velocity of SarCNU in the presence of epinephrine. Points, mean of at least 6 separate experiments with each drug concentration performed in quadruplicate; bars, SE.

between initial rate of SarCNU influx and SarCNU concentration is shown in Figure 3. The nonfacilitated influx rate of SarCNU at high concentrations could not be determined accurately, since, at concentrations of greater than 10 mM SarCNU the intracellular water volume was affected. However, when high concentrations of epinephrine (10 mM) which did not affect intracellular water, 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 ICW/second/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 estimates a K_m of 2.39 ± 0.37 mM and a V_{max} of 236 ± 53 pmol/µl intracellular water/second.

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°C is shown in Figure 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 5 independent experiments was found to be 163 ± 15 µM. Furthermore, the K_i is similar to the K_m of epinephrine

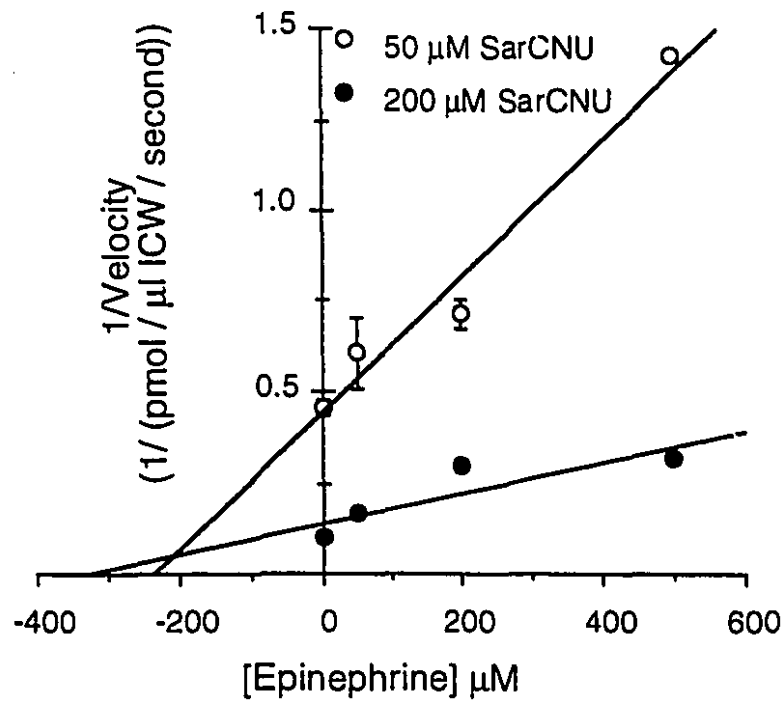


Figure 2.4. Dixon plot of epinephrine inhibition of [^{3}H]SarCNU uptake. The 2 second uptakes of [^{3}H]SarCNU, at concentrations of 50 and 200 µM, in media containing 50 to 500 µM epinephrine were measured in suspension at 37°C, as described in "Materials and Methods." Points, mean of 6 separate experiments with each drug concentration performed in quadruplicate; bars, SE. The plots from top to bottom represent 50 and 200 µM [^{3}H]SarCNU, respectively.

in SK-MG-1 cells which was determined to be 270 μM without correction for diffusion [108].

Metabolism of SarCNU. Thin layer chromatography was used to assess breakdown of 50 μM [^{3}H]SarCNU in the media and intracellularly following a 60 minute incubation, at 37°C, of SarCNU with SK-MG-1 cells. Prior to incubation with cell suspensions, SarCNU was 99% pure with an R_f of approximately 0.81. Following a 60 minute incubation, the SarCNU present in the media was 99% pure and migrated with a similar R_f compared to intact drug. This observation is consistent with the half-life of SarCNU in pH 7.4 media which has been shown to be approximately 5.5 hours [17]. Analysis of cellular lysates in 4 independent experiments, revealed that SarCNU had been metabolized to a limited extent. The percentage of intact SarCNU in cell lysates after a 60 minute incubation was 81.5 ± 4.6%. These results indicate that there is no significant metabolism of SarCNU during the 2 second 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, which is sensitive to SarCNU. Previously, it has been suggested that SarCNU may be transported into human glioma cells by a process which mediates the uptake of catecholamines, and sarcosinamide, the carrier moiety present in SarCNU [108]. These studies provided indirect evidence suggesting that SarCNU enters cells via carrier-mediated uptake as compared to BCNU and other CENUs which enter cells via passive diffusion [109]. Radiolabeled SarCNU was used to directly assess its uptake characteristics in suspension using a modification of "oil-stop" methodology that is highly reproducible.

The uptake of SarCNU in the 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 it enters cells via a non-concentrative 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 have 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 [108]. This system has been shown to mediate the facilitated diffusion of epinephrine and sarcosinamide by a saturable, and sodium independent carrier [108]. 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 [500].

Analysis of the velocity of uptake versus the substrate concentration curve of SarCNU suggests that accumulation of SarCNU is a combination of a saturable (facilitated) and a nonsaturable (nonfacilitated) uptake system. The influence of high concentrations of SarCNU on estimation of the intracellular water space prevented the direct measurement of the rate coefficient of nonfacilitated diffusion. However, since epinephrine is able to inhibit the accumulation of SarCNU to a greater extent than SarCNU itself, epinephrine was utilized to estimate the nonfacilitated diffusion rate of SarCNU. Epinephrine was chosen, since 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 proved to be an estimate of the nonfacilitated diffusion rate of SarCNU. At the theoretical peak plasma concentration of SarCNU, nonfacilitated diffusion is estimated to contribute to 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 [108]. These results are consistent with a common transporter for epinephrine, SarCNU, and sarcosinamide. Previously, Skalski et al. suggested that epinephrine and sarcosinamide share a common catecholamine carrier [108]. 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, 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 of epinephrine and sarcosinamide, greatly enhances the uptake by this transport system [222, 263, 305]. The N-methyl 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 which functions, physiologically, to transport catecholamines. This is the first direct demonstration of a carrier mediated uptake of a CENU. However, other transport systems have been observed for alkylating anti-cancer compounds such as melphalan [504], nitrogen mustard [352], and cyclophosphamide [129]. The transport of SarCNU is similar to that characterized for cyclophosphamide where both compounds appear to enter cells by facilitated diffusion at low concentrations and by a technically nonsaturable (nonfacilitated) mechanism at high doses [129].

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 [108], which enters cells via passive diffusion [109]. Since the catecholamine transporter is found predominantly in tissues of the nervous system [222, 263, 305] and clearly absent in a human lung cancer cell line and human fibroblasts [505], this may account for the increased cytotoxicity of SarCNU in gliomas.

CHAPTER 3

Altered Cytotoxicity of (2-Chloroethyl)-3-sarcosinamide-1-nitrosourea in Human Glioma Cell Lines SK-MG-1 and SKI-1 Correlates with Differential Transport Kinetics

Preface to Chapter 3

Previous work has suggested that human glioma cell line SKI-1 cells, which are resistant to SarCNU compared to SK-MG-1 cells, share a system which may accommodate the uptake of SarCNU in a fashion analogous to that present in SK-MG-1 cells [213]. The work also demonstrated that the ability of SarCNU to inhibit the uptake of sarcosinamide in SKI-1 cells was significantly reduced compared to SK-MG-1 cells. This difference in affinity of SarCNU for the sarcosinamide transporter was suggested to be the potential cause of a reduction of the steady state accumulation in SKI-1 cells versus SK-MG-1 cells. These observations combined with the results of Chapter 2 which described the analysis of SarCNU uptake into SK-MG-1 cells to be a combination of facilitated diffusion, mediated by an epinephrine/sarcosinamide sensitive carrier, and nonfacilitated diffusion suggest that SKI-1 cells may possess an altered SarCNU uptake process. The object of Chapter 3 is to determine if the decreased sensitivity of SKI-1 versus SK-MG-1 cells to SarCNU is a consequence of an alteration in the SarCNU uptake process between the two human glioma cell lines. To this end, the analysis of SarCNU uptake was carried out

to determine the effect of temperature on uptake and steady state of accumulation. Additionally, the saturability of uptake and, where appropriate, the kinetic parameters of SarCNU uptake in SK-MG-1 and SKI-1 cells were examined. Alteration of the steady state levels of a compound may be a consequence of altered efflux between resistant and sensitive cells, so the analysis of efflux of SarCNU was carried out in both cell lines. In addition, the analysis of the cytotoxicity of BCNU and SarCNU was carried out in both cell lines in order to assess the sensitivity/resistance levels to both cytotoxic agents and to correlate these observations with observed and published uptake processes. The most important part of Chapter 3 is to determine whether the uptake of SarCNU into SKI-1 and SK-MG-1 cells is different and to see if the difference of uptake is responsible for mediating altered sensitivity to SarCNU in either cell line. All experiments in Chapter 3 were performed by the candidate excluding the analysis of intracellular metabolism by thin layer chromatography, which was performed by Areti Malapetsa.

SUMMARY

Resistance to (2-chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU), an experimental anticancer compound, was investigated in the chloroethylnitrosourea sensitive, Mer$^-$ SK-MG-1 and resistant, Mer$^-$ SKI-1 human glioma cell lines. The transport of [^{3}H]SarCNU was examined in suspension. The uptake of [^{3}H]SarCNU was found to be temperature dependent in SK-MG-1 and less so in SKI-1. At 37°C, uptake of 50 µM [^{3}H]SarCNU was linear to 4 seconds in both cell lines, with uptake being significantly faster in SK-MG-1 than in SKI-1 under initial rate conditions. There was no significant difference in the rate of influx at 22°C between both cell lines. Equilibrium was approached after 1 minute at 22 and 37°C. At 37°C, steady state accumulation of SarCNU, at 30 minutes, was significantly reduced by 35% in SKI-1 cells compared to SK-MG-1 cells while accumulation was similar at 22°C. In SK-MG-1 cells, at 37°C, uptake of [^{3}H]SarCNU was found to be saturable but uptake in SKI-1 cells was not saturable over a 1000-fold range of concentrations. Analysis of efflux in cells preloaded with 50 µM [^{3}H]SarCNU revealed that the rate of efflux was equivalent in both cell lines, but that the efflux rate was more rapid at 37 compared to 22°C. Metabolism of SarCNU, at 37°C, was not different in either cell line after a 60 minute incubation, as determined by thin layer chromatography. SKI-1 cells as compared to SK-MG-1 cells were 3 fold resistant to SarCNU at 37°C but only 2 fold resistant at 22°C, a temperature at which SarCNU accumulation was similar in both cell lines. The 2 fold resistance at 22°C was similar to that of BCNU at 37 and 22°C. These findings indicate that increased cytotoxicity in SK-

MG-1 cells is associated with a greater accumulation of SarCNU via an epinephrine sensitive carrier which is not detectable in SKI-1 cells. However, part of the chloroethylnitrosourea resistance in SKI-1 cells is not secondary to decreased accumulation.

INTRODUCTION

Resistance to anticancer agents is responsible for the failure to cure many types of cancer. Acquired resistance in cell lines has been demonstrated to be associated with the MDR phenotype [506], elevated GSH levels associated with GST [507], alterations in drug transport [357], and alterations in repair of DNA [508]. The mechanism(s) of innate resistance to chemotherapy is(are) more obscure. It is apparent that several different mechanisms of resistance are involved in this process and that the mechanism may be different depending on the type of anticancer agent and the type of tumor. Resistance to CENUs has been associated with increased drug inactivation by GST [488] and/or increased repair of DNA adducts involving MGMT which prevents the formation of a cytotoxic DNA interstrand cross-link by removing the initial chloroethyl adduct of CENUs formed on O^6-guanine [54, 408, 436]. Additionally, there are alternative mechanisms for repair of CENU-DNA lesions including nucleotide excision repair in MGMT-deficient cells [69] and DNA glycosylase mediated repair of CENU-induced DNA lesions [23, 509].

SarCNU, is a derivative of CENU that contains the amino acid amide group N-methylglycinamide, known as sarcosinamide [17]. Previously, SarCNU has been shown to be more active than the clinically available BCNU in primary glioma cells and glioma cell lines *in vitro* [104] and in human glioma cells intracerebrally implanted into nude mice [105]. In addition, SarCNU is less toxic than BCNU in mice [17] and less myelotoxic in the *in vitro* CFU-C assay with normal human bone marrow [106]. Furthermore, SarCNU is more cytotoxic *in vitro* at its

theoretical PPC of 68 μM compared to BCNU at its clinically achievable PPC of 9 μM [104]. The presence of the sarcosinamide carrier moiety in SarCNU suggests that transport into cells may be altered as compared to BCNU and other clinically available CENUs, which enter cells via passive diffusion [109]. This is supported by the fact that the presence of excess sarcosinamide during *in vitro* cytotoxicity assays increased the ED_{30} of SarCNU, but not that of BCNU, suggesting that SarCNU may enter cells by a carrier mediated mechanism [500]. Initial investigations with [^{3}H]sarcosinamide revealed that sarcosinamide enters cells by the catecholamine, uptake 2 carrier and that SarCNU competitively inhibited this uptake, albeit with a K_i demonstrating ten fold less affinity for the transporter [108].

The human glioma cell line, SK-MG-1, which is sensitive to SarCNU has been used to assess the uptake of SarCNU. Uptake of SarCNU proceeds via a saturable, temperature dependent, and sodium independent catecholamine carrier in SK-MG-1 cells [214]. Additionally, SK-MG-1 and the SarCNU/BCNU resistant human glioma cell line, SKI-1, have been used to assess the uptake of SarCNU indirectly by the use of kinetic analysis of sarcosinamide transport [108, 213]. Analysis of sarcosinamide uptake at 22°C revealed that the K_m of sarcosinamide was 5 fold greater in resistant SKI-1 cells as compared to SK-MG-1 cells. Additionally, Dixon plot analysis revealed that SarCNU competitively inhibits the uptake of sarcosinamide with a K_i 5-fold greater in SKI-1 cells compared to SK-MG-1 cells with the K_i equal to 17.5 and 3.26 mM, respectively. Furthermore, utilizing an insensitive colorimetric assay and non-pharmacological concentrations of 1 mM SarCNU, steady state accumulation of SarCNU at 37°C was found to be 47% reduced in SKI-1

cells versus SK-MG-1 cells, whereas BCNU accumulation was equivalent in both cell lines. The altered accumulation of SarCNU was found to correlate with decreased DNA cross-links in SKI-1. However, DNA repair analysis revealed no MGMT activity, no difference in N-3-methyladenine DNA glycosylase activity, and no difference in the preferential removal of 7-methylguanine or 3-methyladenine by N-3-methyladenine DNA glycosylase in either cell. Northern slot blot analysis revealed no differences between mRNA levels of mdr 1, ERCC-1, or GST-μ. Additionally, SK-MG-1 cells had an 8-fold greater GSH content as compared to SKI-1 cells [213].

In this investigation, SarCNU uptake has been characterized in both cell lines in order to determine whether altered transport of SarCNU is responsible for the difference in sensitivity between SK-MG-1 cells and SKI-1 cells. The effect of temperature on time course of uptake, steady state of accumulation, and efflux of SarCNU was examined for both cell lines at a concentration in the range of the theoretical PPC of SarCNU. Finally, intracellular metabolism of SarCNU was analyzed in both glioma cell lines.

MATERIALS AND METHODS

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

Materials. (±)epinephrine HCl was obtained from Sigma Chemical Co. (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. [17]. Briefly, [^{3}H]sarcosinamide was prepared by use of a catalyzed exchange reaction with high specific activity tritiated water and the crude preparation 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 (2-chloroethyl)-3-[^{3}H]sarcosinamide-1-nitrosourea which was purified by HPLC. 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 sodium acetate pH 4.0:water (4:2:1) with an R_f value of 0.81. [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. McCoy's 5A modified medium, fetal calf serum, and Dulbecco's phosphate buffered saline (D'PBS) were supplied by Canadian Life Technologies (GIBCO, Montréal, Québec, Canada). Versilube F-50 silicone oil was from Nessa Products (Montréal, Québec, Canada). Dextrose, and NaCl were from Fisher Laboratories.

Cell culture. SK-MG-1 and SKI-1 cells were established from untreated human glioma specimens. SKI-1 cells were generously provided by Dr. J. Shapiro (Barrow's Neurological Institute, Phoenix, Arizona). SK-MG-1 cells 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, Québec, Canada) in a humidified 5% CO_2 atmosphere at 37°C. 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 x g for 5 minutes, washed twice in PAG and resuspended in PAG to either $4x10^6$ or $2x10^6$ cells/ml, as needed.

Transport Experiments. A modified version of "oil-stop" methodology was used to assay transport of [^{3}H]SarCNU, at a 50 µM concentration (specific activity of 6.84 µCi/µmol) in the glioma cells, in suspensions, at 0, 22, and 37°C as described previously [136, 214]. The accumulation of SarCNU in cells is expressed as cell to medium ratio which describes the distribution of SarCNU in 1 µl of the intracellular water space (ICW) and the extracellular medium. The ICW was determined, after mixture of cell suspension and unlabeled permeant with tritiated water and [carboxyl-^{14}C]inulin by centrifugation of cells through silicone oil, as described previously [214, 503]. [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 cells 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 of uptake was performed as described above, except various concentrations of osmotically adjusted SarCNU (final specific activity from 6.84 µCi/µmol to 0.00684 µCi/µmol) was 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 was 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 2 seconds (minus zero-time), during the linear phase of influx [214].

Measurement of efflux of [^{3}H]SarCNU. Efflux experiments were performed by a modification of a previously described technique [510]. Briefly, cells in suspension ($2x10^6$ cells/ml), at 22°C, were preincubated for 15 minutes, and treated with [^{3}H]SarCNU, at a 50 µM concentration, for 20 minutes. The cells were loaded at 22°C to ensure that both SKI-1 cells and SK-MG-1 cells achieve a similar cell to medium ratio prior to initiation of efflux. Several 400 µl aliquots were removed prior to and during the preparation of cells for the

efflux experiments and transport terminated by centrifugation through versilube oil in order to analyze cell to medium ratio, as previously described [214]. The remaining preloaded cells were aliquoted (400 µl) into 1.5 ml microcentrifuge tubes and centrifuged for 5 minutes at 300 x g. Medium was aspirated, leaving approximately 10 µl of loading media, and cell pellet incubated at 22°C until needed. Immediately before use, each cell pellet was gently disrupted. To initiate efflux of [^{3}H]SarCNU, the cell suspension was diluted 40-fold by the addition of 400 µl of PAG, at either 22 or 37°C, to the concentrated cell suspension. At the indicated time points, efflux was terminated by separating the cells from the media by centrifugation through versilube oil, and cell pellet processed, as described previously [214]. An aliquot of the medium was removed and the [^{3}H]SarCNU content analyzed to assure that the 40-fold dilution was obtained. Every time point, in each individual experiment, was performed in quadruplicate.

The ICW and zero-time analysis was performed by the same procedure as for the analysis of transport. Medium contamination of cell pellets was subtracted from every experimental determination, as described. The cell to medium ratio was calculated from the loaded cells removed prior to the efflux procedure to estimate the total amount of [^{3}H]SarCNU associated with the cells (maximal initial concentration). This estimate was counted as 100% of loaded cell dpm, and subsequent efflux points are described as percent of dpm radiolabel retained.

Measurement of Cytotoxicity. Analysis of the cytotoxicity of SarCNU and BCNU was performed by a modification of the sulforhodamine B

(SRB) colorimetric anticancer-drug screening assay [511, 512]. Briefly, SK-MG-1 and SKI-1 cell lines were plated at a density 1100 and 1800 cells per well, respectively, on a flat bottom 24-well plate. Following a 16 hour incubation, cultures were washed once with D'PBS, and then treated for 60 minutes with various concentrations of SarCNU or BCNU in PAG. Controls contained appropriate quantities of diluent. At the end of the 60 minute incubation, the PAG was aspirated and cultures feed with 2.0 ml of McCoy's 5A medium and then incubated for 7 days. Cultures were fixed to the plastic substratum by removing the culture medium and gently adding 1.0 ml of a 10% TCA and 0.9% NaCl solution. The cultures were incubated for 1 hour at 4°C and then washed five times with water to remove TCA. Plates were air dried and then stained with a 0.4% SRB dissolved in 1.0% acetic acid for 30 minutes. SRB was then removed and cultures were quickly rinsed five times with 1.0% acetic acid to remove unbound dye. The cultures were air dried and bound dye solubilized with 2 ml of 10 mM unbuffered Tris base (pH 10.5). OD was read using the Hewlett Packard 8451A Diode Array Spectrophotometer at 500-550 nm. The wavelength utilized was varied in order to obtain maximum sensitivity as described [511]. IC_{90} values were obtained by exponential curve fit of the linear portion of the cytotoxicity curve utilizing CA-Cricket Graph III version 1.01 (Computer Associates International, Inc., Islandia, New York).

Metabolism of [^{3}H]SarCNU. Samples obtained from transport assays were utilized in these experiments. Cell suspensions ($2x10^6$ cells/ml) were incubated with [^{3}H]SarCNU, at a 50 µM concentration (specific activity 6.84 µCi/µmol), for 60 minutes at 37°C 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°C 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.

Calculations. Statistical analysis of influx rate, and cytotoxicity values were performed by the two tailed t test utilizing StatView 512+ (BrainPower, Inc., Calabasas, California).

RESULTS

Time Course of Uptake of [^{3}H]SarCNU at Different Temperatures. A time course, performed at different temperatures, of the uptake of [^{3}H]SarCNU, at a 50 μM concentration, in SK-MG-1 cells and SKI-1 cells is shown in Fig. 1. At 37°C, uptake of SarCNU was linear to 4 seconds in both cell lines and begins to reach equilibrium after 1 minute. Additionally, analysis of the initial rate of influx at SarCNU concentrations of as great as 50 mM were found to be linear to 4 seconds (results not shown). The initial rate of SarCNU influx at 2 seconds was found to be more temperature dependent in SK-MG-1 cells than SKI-1 cells. The values for SK-MG-1 were 1.25 ± 0.50, 3.75 ± 0.25, and 6.25 ± 0.5 pmol/μl ICW/sec at 0, 22, and 37°C, respectively. The values for SKI-1 were 2.75 ± 0.75, 3.00 ± 0.75, and 4.50 ± 0.25 pmol/μl ICW/sec at 0, 22, and 37°C, respectively. All subsequent kinetic experiments were carried out at 2 seconds to approach the initial rate of uptake conditions and to minimize the effects of efflux because the cell to medium ratio, minus zero-time, was below 0.3 at this point [123].

Comparison of uptake of [^{3}H]SarCNU, at a 50 μM concentration, in SK-MG-1 cells and SKI-1 cells at 37°C demonstrates that the rate of influx of SarCNU into SK-MG-1 cells is greater than the influx rate into SKI-1 cells under initial rate conditions (Fig. 1). The difference between the rates of accumulation at 37°C are statistically significant at 2 seconds (6.25 ± 0.5 pmol/μl ICW/sec for SK-MG-1 cells versus 4.50 ± 0.25 pmol/μl ICW/sec for SKI-1 cells, $p = 0.0227$). However, comparison of the initial rate of SarCNU influx at 22°C does

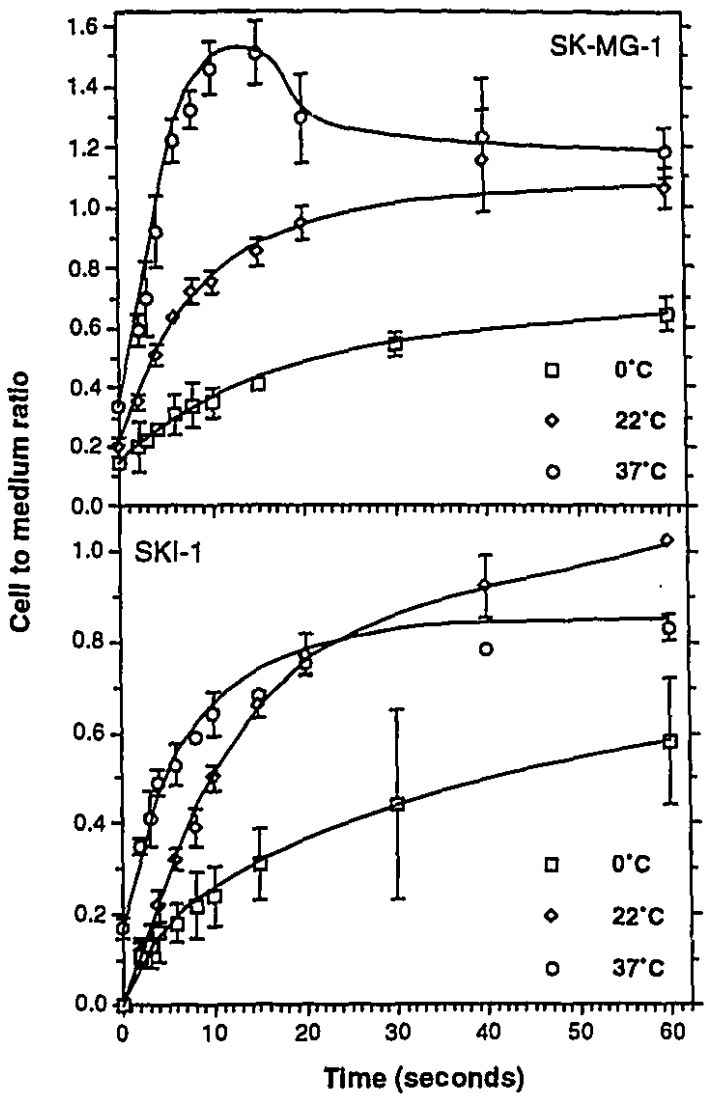


Figure 3.1. Time course of uptake of [^{3}H]SarCNU, at a 50 μM concentration, by SK-MG-1 cells and SKI-1 cells in suspension at 0, 22, and 37°C. The uptake, in suspension, is expressed as cell to medium distribution ratio as described in "Materials and Methods." The zero-time cell to medium distribution ratios for SK-MG-1 were 0.150 ± 0.002, 0.20 ± 0.03, and 0.34 ± 0.02 for 0, 22, and 37°C, respectively. The zero-time cell to medium distribution ratios for SKI-1 were 0 for 0 and 22°C, and 0.17 ± 0.02 for 37°C. Points, mean of at least 4 separate experiments, with each time point performed in quadruplicate; bars, SE.

not demonstrate significant differences between the two cell lines (Fig. 1).

Steady State Accumulation of [^{3}H]SarCNU. Following a 30 minute incubation with 50 μM [^{3}H]SarCNU, the cell to medium ratio was determined in SK-MG-1 cells and SKI-1 cells at 22 and 37°C (See Appendix 4). Accumulation of SarCNU in SK-MG-1 cells, expressed as cell to medium ratio is 1.08 ± 0.03 at 22°C and 1.28 ± 0.04 at 37°C. In SKI-1 cells accumulation of SarCNU is 0.92 ± 0.02 and 0.83 ± 0.03 at 22 and 37°C, respectively. There is a significant 35% ($p=0.0001$) decrease in intracellular concentration of SarCNU at 37°C while accumulation of SarCNU is similar at 22°C in SKI-1 cells as compared to SK-MG-1 cells. These results, utilizing 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 [213]. Additionally, the intracellular concentration of SarCNU in SK-MG-1 is significantly reduced by 16% at 22 versus 37°C ($p=0.0032$). However, in SKI-1 cells the intracellular concentration of SarCNU is significantly reduced by 10% at 37 versus 22°C ($p=0.033$).

Kinetic Analysis of [^{3}H]SarCNU Uptake. Since the initial rate of influx of SarCNU was more rapid in SK-MG-1 cells than SKI-1 cells at 37°C, analysis of the kinetics of uptake was carried out. The relationship between cell to medium ratio and SarCNU concentration for SK-MG-1 cells and SKI-1 cells is shown in Fig. 2. In SK-MG-1, as the drug concentration increased from 50 μM to 7.5 mM the cell to medium

ratio decreased (Fig. 2). Thereafter, there was no further decrease in SarCNU distribution up to a 50 mM concentration (Fig. 2 and results not shown). In SKI-1, however, there was no observable decrease in cell to medium ratio of SarCNU when drug concentrations were increased from 50 μM to 50 mM (Fig. 2 and results not shown). Furthermore, a 10 mM concentration of SarCNU was able to significantly inhibit the initial rate of influx of [^{3}H]SarCNU, at a 50 μM concentration, by 24% in SK-MG-1 cells [214] but not in SKI-1 cells (control velocity of 5.9 ± 0.83 versus 5.4 ± 0.63 pmol/μl ICW/sec for 10 mM SarCNU in SKI-1 cells). At 22°C, there was no evidence of a saturable transport system in either cell line (control cell to medium ratio at 4 seconds was 0.17 ± 0.01 and 0.24 ± 0.05 at 50 μM [^{3}H]SarCNU for SKI-1 and SK-MG-1, respectively, versus 0.18 ± 0.02 and 0.22 ± 0.06 at 10 mM [^{3}H]SarCNU for SKI-1 and SK-MG-1, respectively).

Efflux of [^{3}H]SarCNU. Comparison of efflux in SK-MG-1 cells and SKI-1 cells preloaded with [^{3}H]SarCNU, at a 50 μM concentration, at 22°C is shown in Fig. 3. Both cell types were preloaded at 22°C in order to circumvent problems associated with differential SarCNU accumulation at 37°C. At 37°C, the efflux of SarCNU is rapid and begins to plateau at 1 minute with SK-MG-1 retaining 26 ± 3% of radiolabel and SKI-1 retaining 23 ± 1% of radiolabel. At 30 minutes, the percentage of intracellular SarCNU transferred out of SK-MG-1 and SKI-1 is approximately 92% for both. Efflux, at 22°C, is similarly reduced in both cell lines as compared to that at 37°C. However, at 22°C, efflux out of these cells is still rapid with 33 ± 1% and 26 ± 3%

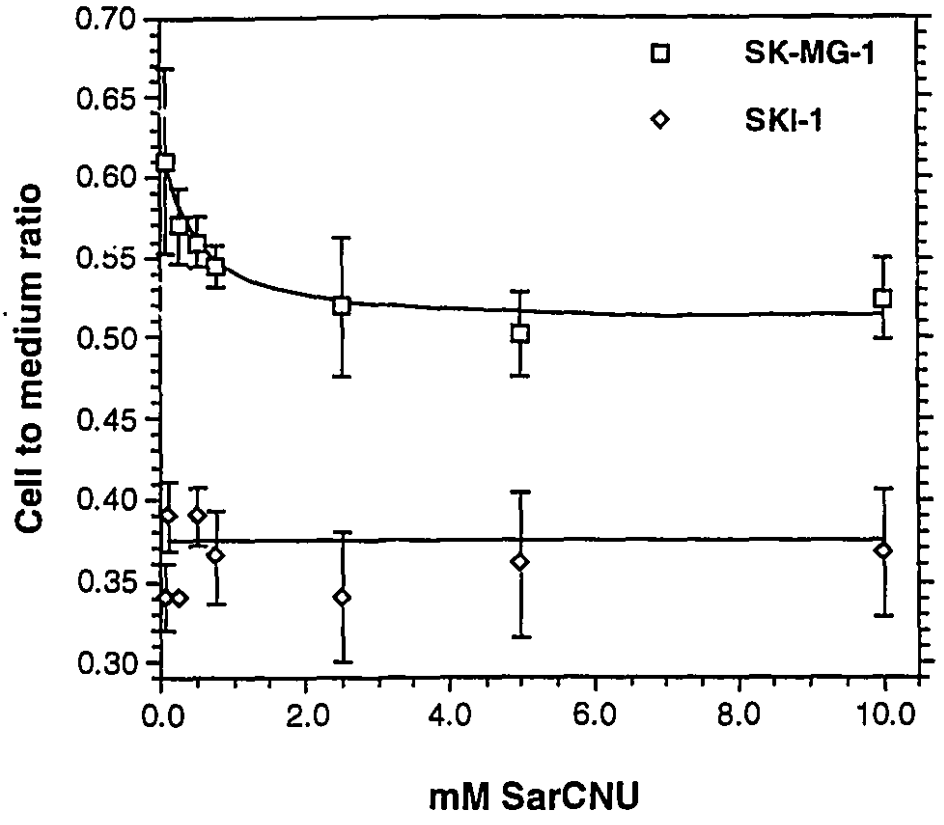


Figure 3.2. Comparison of the uptake of [^{3}H]SarCNU, at 2 seconds, versus concentration of unlabeled SarCNU in SK-MG-1 cells and SKI-1 cells at 37°C. The uptake, in suspension, is expressed as cell to medium distribution ratio as described in "Materials and Methods." Points, mean of at least 6 separate experiments, with each time point performed in quadruplicate; bars, SE.

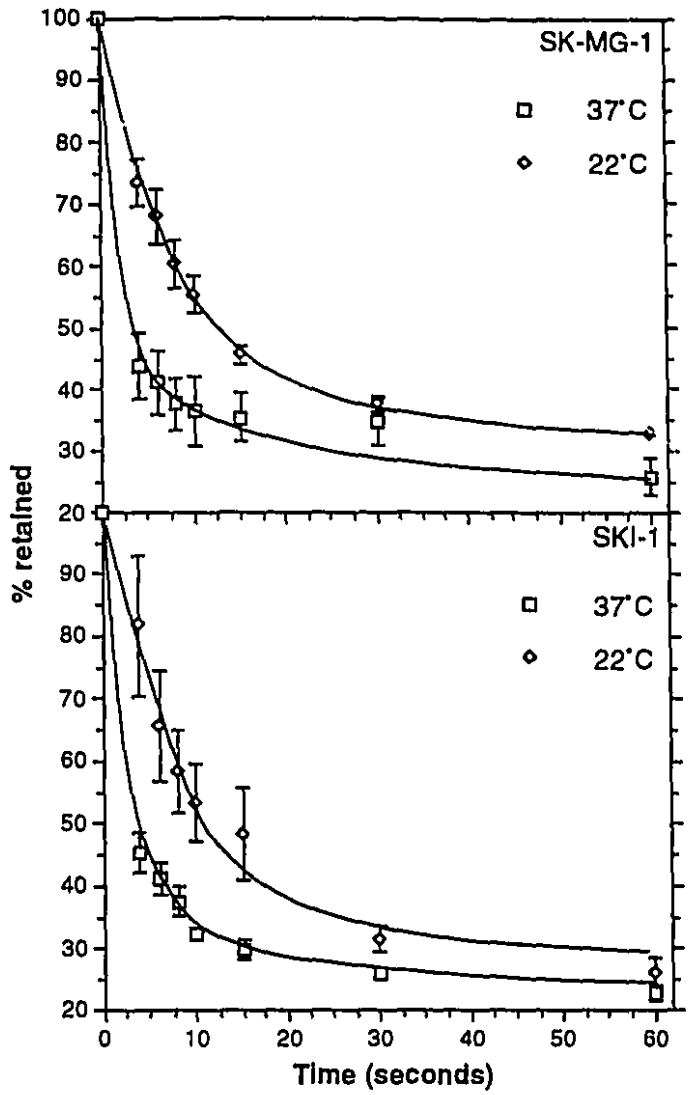


Figure 3.3. Comparison of the time course of efflux of [^{3}H]SarCNU, at a 50 μM concentration, by SK-MG-1 cells and SKI-1 cells at 22 and 37°C. The efflux, in suspension, is expressed as percent radiolabel retained as described in "Materials and Methods." Points, mean of at least 4 separate experiments, with each time point performed in quadruplicate; bars, SE.

of initial SarCNU retained at 1 minute in SK-MG-1 and SKI-1, respectively (Fig. 3).

Cytotoxicity of SarCNU and BCNU. Comparison of the IC_{90} cytotoxicity values of SarCNU and BCNU in CENU resistant SKI-1 cells versus sensitive SK-MG-1 cells are shown in Table 1. At both temperatures, the concentration of CENU required to achieve an IC_{90} value is significantly greater in SKI-1 cells compared to SK-MG-1 cells, consistent with the observation that SKI-1 cells are more resistant to CENU treatment than SK-MG-1 cells [104]. The 3 fold difference in the sensitivity of SKI-1 cells versus SK-MG-1 cells to SarCNU at 37°C is significantly greater than the 2 fold difference at 22°C ($p=0.0001$, Fig. 4). The fold difference in sensitivity between SKI-1 cells and SK-MG-1 cells to SarCNU at 22°C and to BCNU at 22 and 37°C are all similar (see Appendix 5 for BCNU cytotoxicity), approximately a 2 fold difference.

Metabolism of SarCNU. Thin layer chromatography was used to assess breakdown of [^{3}H]SarCNU, in the media and intracellularly, following a 60 minute incubation of 50 μM SarCNU with either SK-MG-1 cells or SKI-1 cells. Prior to incubation with cell suspensions, SarCNU was 99% pure with an R_f of approximately 0.81. Following a 60 minute incubation, the SarCNU present in the media of both cell lines was 99% pure and migrated with a similar R_f compared to intact drug. This observation is consistent with the half-life of SarCNU in pH 7.4 media which has been shown to be approximately 5.5 hours [17]. Analysis of cellular

Table 3.1 Effect of temperature on the cytotoxicities of SarCNU and BCNU in SK-MG-1 cells and SKI-1 cells.

SK-MG-1 and SKI-1 cells were incubated with dilutions of SarCNU or BCNU for 60 minutes at the indicated temperature. Cytotoxicity values were determined by the SRB protein dye binding assay, seven days post-treatment, as described in "Materials and Methods."

Compound	Temperature (°C)	Cell line	n[a]	IC_{90} (μM)[b]	p[c]	Fold Difference[d]
SarCNU	37°	SK-MG-1	15	120 ± 10	0.0001	
		SKI-1		455 ± 20		3.1 ± 0.1
	22°	SK-MG-1	9	470 ± 45	0.0004	
		SKI-1		975 ± 100		2.1 ± 0.2
BCNU	37°	SK-MG-1	11	125 ± 20	0.0018	
		SKI-1		220 ± 20		2.0 ± 0.2
	22°	SK-MG-1	6	220 ± 30	0.023	
		SKI-1		325 ± 30		1.6 ± 0.1

[a] The number of individual experiments perfcrmed.

[b] IC_{90}, 90% inhibitory concentration.

[c] Two-tailed t test determining the significance between the IC_{90} values.

[d] Average of the fold difference of IC_{90} values between SKI-1 cells and SK-MG-1 cells in individual experiments.

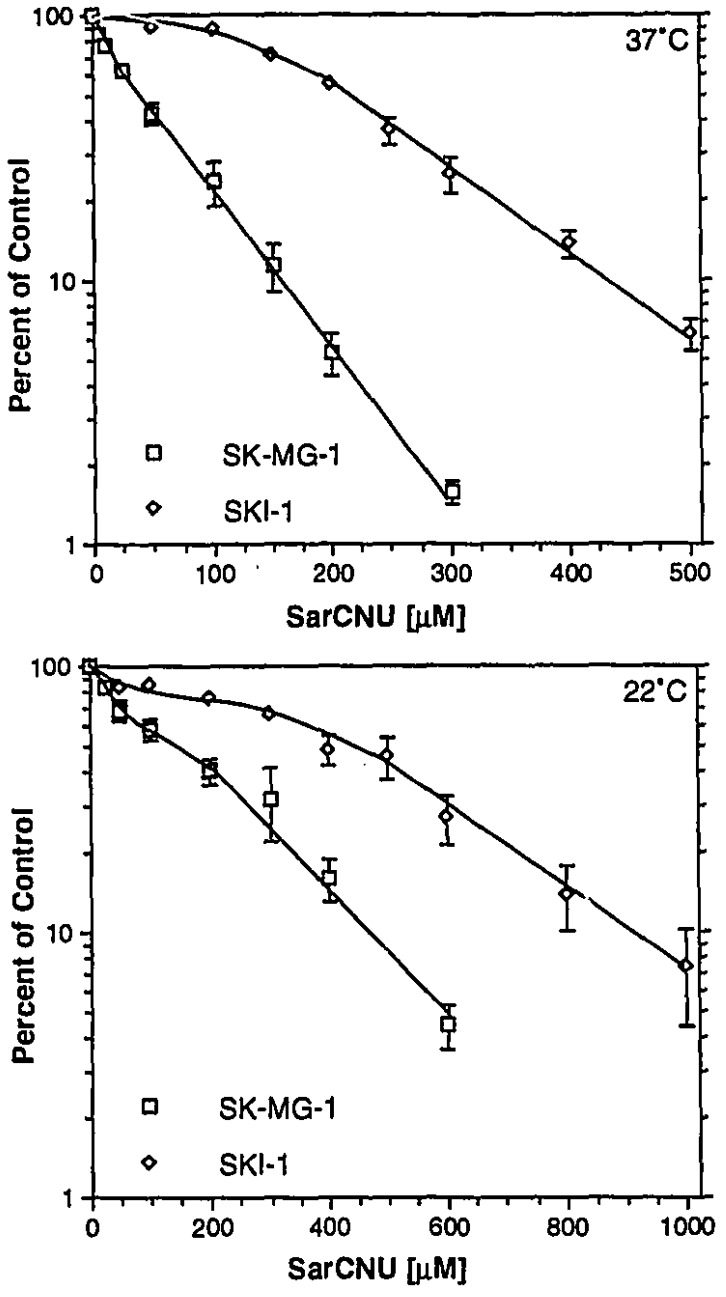


Figure 3.4. Comparison of the cytotoxic activity of SarCNU in SK-MG-1 cells and SKI-1 cells at 22 and 37°C in the SRB assay. The percentage of control SRB dye binding was plotted against the concentrations in µM of SarCNU. Points, mean of 15 separate experiments for 37°C, and mean of 9 separate experiments for 22°C, with each drug concentration performed in quadruplicate; bars, SE.

lysates in 4 independent experiments, revealed that SarCNU had been metabolized to a limited extent in both cell lines. The percentage of intact SarCNU in cell lysates after a 60 minute incubation was 81.5 ± 4.6% in SK-MG-1 and 86.1 ± 1.1% in SKI-1. These results indicate that there is no significant difference in the ability of either cell line to metabolize SarCNU.

DISCUSSION

This investigation was carried out to define the mechanism of resistance to SarCNU, an experimental drug, in two previously untreated human glioma cell lines. The steady state accumulation of SarCNU was examined and was found to be significantly reduced in SKI-1 cells as compared to SK-MG-1 cells at 37°C. At 22°C, influx of SarCNU was not saturable and drug accumulation was similar in both cell lines. However, comparison of the initial rate of influx at 37°C between SK-MG-1 cells and SKI-1 cells demonstrated an increased influx of SarCNU in SK-MG-1 cells versus SKI-1 cells. The altered rate of initial influx of SarCNU at 37°C is consistent with the observed 35% reduced steady state accumulation in SKI-1 cells compared to SK-MG-1 cells at 37°C. Analysis of the kinetics of SarCNU influx at 37°C has revealed that uptake in SK-MG-1 cells was saturable, and sodium independent [214], whereas, uptake in SKI-1 cells was found to be non-saturable. The saturable component of SarCNU influx, in SK-MG-1 cells, was shown to be inhibited competitively by epinephrine (K_i of approximately 165 μM) and to obey Michaelis-Menten kinetics (0.05-10.0 mM) with a K_m and V_{max} equal to approximately 2.4 mM and 240 pmol/μl ICW/second, respectively [214]. These features are consistent with a carrier mediated transport of SarCNU, at 37°C, in SK-MG-1 cells and not SKI-1 cells. The observation that SarCNU enters SK-MG-1 cells but not SKI-1 cells via a saturable carrier is somewhat different to our previous studies using the analysis of sarcosinamide uptake to assess the transport of SarCNU [108, 213]. In these studies, with the use of Dixon plot analysis, it was found that SarCNU was able to competitively

inhibit sarcosinamide uptake with SK-MG-1 cells having a K_i 5-fold reduced compared to SKI-1 cells at 22°C. Our previous results have demonstrated that the K_i of SarCNU inhibition of sarcosinamide uptake in SKI-1 cells was greater than 10 mM, and in these experiments, equivalent and greater concentrations were found to affect the measurement of intracellular water and therefore are not accurate in the assessment of transport kinetics [214]. The presence of a saturable carrier in SK-MG-1 cells and a non-detectable carrier in SKI-1 cells is consistent with the increased initial rate of uptake observed in SK-MG-1 cells versus SKI-1 cells at 37°C.

It is conceivable that the altered accumulation of SarCNU could be as a consequence of having an altered mechanism of efflux in either SK-MG-1 cells or SKI-1 cells. This possibility is unlikely since it was determined that both cell lines have equal rates of efflux when assessed at 22 and 37°C. The uptake and efflux of SarCNU in both cell lines is equivalent at 22°C, thus leading to similar values for the steady state accumulation of SarCNU. However, at 37°C, the initial rate of influx of SarCNU is greater in SK-MG-1 cells than SKI-1 cells with efflux being equivalent resulting in a greater SarCNU accumulation in SK-MG-1 cells. In fact, the resistance between SKI-1 cells and SK-MG-1 cells to SarCNU significantly decreased from 3 fold at 37°C to 2 fold at 22°C. The 2 fold resistance that remains is similar to the 2 fold resistance to BCNU between these cell lines at 22 and 37°C. There are several examples of resistance related to decreased cellular uptake of alkylating agents such as melphalan, nitrogen mustard, and cis-platin [352, 504, 513]. However, to date, resistance related to

alterations in accumulation of CENUs has not been demonstrated, as in the case of SarCNU.

A 2 fold resistance to BCNU has been observed in SKI-1 cells compared to SK-MG-1 cells that can not be explained by altered accumulation since accumulation of BCNU is similar in both cell lines [108, 213]. Previously, it has been shown that there are no differences in MGMT activity, N-3-methyladenine DNA glycosylase activity, and mRNA levels of mdr 1, ERCC-1, or GST-μ [213]. Analysis of the time course of formation and removal of DNA cross-links in SK-MG-1 cells and SKI-1 cells demonstrated a reduction of the total number of DNA cross-links in SKI-1 cells versus SK-MG-1 cells [213], which can be secondary to either decreased intracellular accumulation, and/or alteration in the kinetics of cross-link formation and removal. Recent results, using the analysis of CENU resistant (SKI-1 and T98G) and sensitive (SK-MG-1) glioma cell lines by Northern blot, have demonstrated an increased expression of ERCC-2 and metallothionein, suggesting that there may be other mechanisms of CENU resistance [514]. Consequently, plasmid reactivation assays will be utilized to better define DNA repair, in SK-MG-1 and SKI-1, in order to determine if the 2 fold BCNU resistance in SKI-1 is associated with increased repair of CENU-adducted DNA.

The present study demonstrates that SarCNU has increased cytotoxicity in SK-MG-1 cells that is associated with a carrier mediated rapid initial uptake not detectable in the resistant SKI-1 cells. This epinephrine sensitive, carrier mediated uptake 2 of SarCNU may, therefore, be responsible for its increased cytotoxicity in glioma cells as compared to BCNU and other clinically available CENUs which

enter cells by passive diffusion [109, 214]. The catecholamine (uptake 2) transporter which is inhibited by O-methylated catecholamines, corticosteroids, and ß-haloalkylamines [515] is clearly different from uptake$_1$ and exists in various tissues such as myocardium, salivary glands, and vascular smooth muscle [326]. Recently, the presence of the catecholamine (uptake 2) carrier has been described in a human renal carcinoma cell line [326] suggesting that other tumors may be selectively sensitive to SarCNU.

CHAPTER 4

Characterization of the catecholamine extraneuronal uptake$_2$ carrier in human glioma cell lines SK-MG-1 and SKI-1 in relation to SarCNU selective cytotoxicity.

Preface to Chapter 4

Chapter 3 described the comparison of SK-MG-1 and SKI-1 cells with respect to SarCNU uptake. The analysis showed that SKI-1 cells did not demonstrate the presence of a SarCNU transporter but instead have a technically nonsaturable uptake of SarCNU consistent with passive diffusion. This lack of a saturable carrier system for SarCNU in SKI-1 cells was consistent with a reduced initial rate of SarCNU influx and reduced steady state levels. The absence of a SarCNU carrier in SKI-1 cells was suggested to be the cause of decreased sensitivity to SarCNU since SK-MG-1 cells have increased uptake and steady state levels of SarCNU associated with the SarCNU transporter. In Chapter 2, the carrier-mediated uptake of SarCNU into SK-MG-1 cells was suggested to occur via an epinephrine sensitive system thought to be the catecholamine extraneuronal uptake$_2$ transporter. There was no direct demonstration as to the identity of the SarCNU transporter. To this date, a direct demonstration of the presence of the uptake$_2$ transporter in human glioma cells has not been carried out. The purpose of Chapter 4 is to address the hypothesis that the catecholamine uptake$_2$ transporter is present in SK-MG-1 but not SKI-1

human glioma cells and that the transport of SarCNU through the uptake$_2$ transporter is responsible for the increased sensitivity of SK-MG-1 cells to SarCNU compared to SKI-1 cells which lack the carrier. Prior to the characterization of the epinephrine transporter, the ability of sarcosinamide and epinephrine, both inhibitors of SarCNU uptake ([108] and Chapter 2), to inhibit SarCNU uptake into SK-MG-1 and SKI-1 cells was analyzed to further confirm the lack of SarCNU transport in SKI-1 cells. In addition, the analysis of the time-course of SarCNU uptake/accumulation over 60 minutes was performed to address the suggestion that reduction of SarCNU toxicity in SK-MG-1 cells by sarcosinamide is a consequence of inhibition of SarCNU transport leading to reduced accumulation and, consequently, reduced cytotoxicity [500]. The majority of the work in this chapter is in order to identify the uptake$_2$ transporter directly and to ask if the uptake$_2$ transporter is responsible for the transport of SarCNU. Norepinephrine was utilized to specifically demonstrate the presence or absence of the uptake$_2$ transporter, as it is a naturally occurring substrate of this system [218]. Since uptake$_2$ is known to be involved in a metabolic cascade of catecholamines [218], the metabolism of norepinephrine was analyzed, in the presence and absence of inhibitors of the catecholamine metabolic pathway, to determine the extent of norepinephrine metabolite formation both extracellularly and intracellularly. Following metabolism analysis, the examination of norepinephrine uptake was carried out in both SK-MG-1 and SKI-1 cells to determine the effect of temperature on uptake, the saturability and, where appropriate, the kinetic parameters of norepinephrine uptake. Additionally, the ability of desipramine, a potent uptake$_1$ inhibitor,

and a variety of uptake$_2$ inhibitors to inhibit the uptake of SarCNU and norepinephrine was examined to determine the chemical specificity and similarities of both uptake processes. Since both catecholamines (epinephrine and norepinephrine) and SarCNU may compete for the same uptake carrier mechanism, the method of Dixon [128] was utilized, where appropriate, in order to determine if the compounds were able to competitively inhibit each others' transport. Furthermore, the effects of K^+ and ouabain, a Na^+/K^+ ATPase inhibitor, on the uptake of both SarCNU and norepinephrine were examined with the reasoning that they are known to alter uptake of catecholamines through the uptake$_2$ transporter. The most important part of Chapter 4 is to determine if the extraneuronal uptake$_2$ transporter is present in a human glioma cell line and is it responsible for the carrier-mediated uptake of SarCNU in SK-MG-1 cells which is absent in SKI-1 cells. All experiments in Chapter 4 were performed by the candidate excluding portions of the analysis of intracellular and extracellular metabolism by cascade column chromatography, which were performed by Areti Malapetsa, James Barton, and Daniela Marcantonio.

SUMMARY

Transport of (2-chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) was investigated in 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 seconds 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 SKI-1 cells. In SK-MG-1 cells, [^{3}H](-)-norepinephrine influx was found to be competitively inhibited by (-)-epinephrine (K_i = 111 ± 6 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 significantly reduce 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.

INTRODUCTION

More than half of the patients that develop malignant brain tumors present with malignant high-grade astrocytomas [87]. The current standard therapy for patients with high grade astrocytomas involves surgical resection followed by radiotherapy. The addition of chemotherapy to this therapy has only marginally improved the 1 year survival rate of patients compared to surgical resection and radiotherapy. It has recently been determined that the addition of chemotherapy to surgery and radiation improved the median survival duration of patients from 9.4 to 12 months [93]. The chloroethylnitrosoureas (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 [87]. BCNU is currently one of the most active CENUs [87, 516-518].

The CENUs result in dose limiting toxicity of delayed cumulative myelosuppression [509]. 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 [17]. Previously, SarCNU has been shown to be more active than BCNU in primary gliomas plus glioma cell lines *in vitro* [104] and in a human glioma cell line intracerebrally implanted into nude mice [105]. Moreover, SarCNU is less toxic than BCNU in mice [17] and less myelotoxic in the *in vitro* colony forming unit (cell) assay with normal human bone marrow [106]. Furthermore, SarCNU is more toxic *in vitro*, to primary human gliomas,

at its theoretical PPC of 68 μM compared with BCNU at its clinically achievable PPC of 9 μM [104].

The clinically available CENUs which include BCNU, CCNU and methyl CCNU all enter cells via passive diffusion [109]. 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 increased the 70% inhibitory concentration of SarCNU but not that of BCNU [500]. Initial investigations with [^{3}H]sarcosinamide suggested that sarcosinamide enters cells through an epinephrine sensitive carrier system and that SarCNU was able to competitively inhibit this uptake [108].

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 [104, 215]. 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 [214]. However, influx of [^{3}H]SarCNU into SKI-1 cells was found to occur through passive diffusion [215]. The steady state accumulation of SarCNU, at 37°C, was found to be significantly increased by 54% in SK-MG-1 compared to SKI-1. This difference in accumulation, at 37°C, 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 minute 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 and not in SKI-1 cells [215]. 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) [222]. The perisynaptic location of astrocytes is thought to indicate that they may play a role in transmitter reuptake at the neuronal junction [223, 224, 226, 236]. Since uptake into extraneuronal sites is followed by metabolism by MAO and COMT, the role astroglial cells play in the reuptake of catecholamines has not been well defined due to insufficient experimental data [218, 222]. 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 [305]. 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 extensively characterized [226, 227].

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 [17]. It was dissolved in 0.001 M citrate buffer, pH 4.0, aliquoted, and stored at -20°C.

Materials. (±)-Epinephrine HCl, (±)-norepinephrine HCl, (-)-epinephrine bitartrate, (-)-norepinephrine bitartrate, sarcosinamide HCl, papaverine HCl, cimetidine HCl, and desipramine HCl were obtained from Sigma Chemical Co. 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. (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 [17]. 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 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. The radiochemical purity was greater than 95%, as determined by the cascade column chromatographic technique of Graefe et al [519]. [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. McCoy's 5A modified medium, fetal bovine serum, and Dulbecco's phosphate buffered saline (D'PBS) were supplied by Canadian Life Technologies (GIBCO, Montréal,

Québec, Canada). Versilube F-50 silicone oil was from Nessa Products (Montréal, Québec, Canada). Dextrose, sodium acetate, HCl, KCl, and NaCl were from Fisher Laboratories. Alumina and Dowex 50Wx4 were purchased from Aldrich. Disprocynium-24 was a kind 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 [215]. Confluent monolayers of cells were washed once with PAG and harvested with a rubber policeman. Cell suspensions were centrifuged at 300 x g for 5 minutes, washed twice in PAG and resuspended in PAG to either $4x10^6$ or $2x10^6$ 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 0.1 µM, 50 µM or 20 mM concentration (specific activity, 11.9 Ci/mmol, 23.8 mCi/mmol or 59.5 µCi/mmol, respectively), to 1.0 ml of cell suspension ($4x10^6$ cells/ml). The suspensions were incubated for 5, 10, and 15 seconds at 37°C. At the appropriate time, 10 ml of ice cold PBS was added and the suspensions were centrifuged at 300 g for 2 minutes at 4°C. Immediate addition of ice cold PBS was utilized for the "zero-time" metabolism point. Media 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 minutes at 4°C. Following aspiration, the cell pellets were lysed with 100 µl of 0.2 M HCl:ethanol (9:1). All samples were stored at -20°C and

chromatographic analysis was done within 24 hours. Prior to chromatography, 900 µl of 2 M Na 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 Brinkman Eppendorf microcentrifuge for 3 minutes at room temperature. The supernatant was then applied to the cascade chromatography technique. The contents of [^{3}H]norepinephrine and its metabolites in media and cell lysates were determined with the cascade column chromatography technique of Graefe et al [519, 520]. Calibration of the column technique was performed with all metabolites as discussed in Appendix 6.

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 (specific activity, 23.8 mCi/mmol) in the glioma cells, in suspension, at 0 and 37°C, as described previously [136, 214]. 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 intracellular water space (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 [214, 215]. [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 cells 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 various concentrations of osmotically adjusted norepinephrine (final specific activity, 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 was 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 seconds (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 [214]. Analysis of the effect of 100 mM KCl replacing NaCl in isoosmotically adjusted PAG was carried out, as previously utilized [320]. Briefly, cells were pretreated for 15 minutes at 37°C, prior to examining the uptake of [^{3}H]norepinephrine, at a concentration of 25 μM, at "zero-time" and 5 seconds, as described [214, 215]. The ICW was determined, individually, for each pretreatment, as above.

SarCNU Transport Experiments. Kinetic analysis and inhibition of influx, at 2 seconds, of [^{3}H]SarCNU, at concentrations of 50 μM and 200

μM (final specific activity, 6.84 μCi/μmol and 1.71 μCi/μmol), was performed at 37°C, as described above, except 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 (specific activity, 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, California)

RESULTS

Inhibition of [^{3}H]SarCNU accumulation. It has been observed that the presence of excess sarcosinamide during 60 minute SarCNU cytotoxicity assays reduced effectiveness of SarCNU in SK-MG-1 cells [500]. 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 (Figure 1). The area under the curve of total intracellular SarCNU exposure over time is reduced significantly by 9% ($p = 0.0362$, 86 and 78 cell to medium ratio units for influx in the absence and presence of sarcosinamide, respectively). Additionally, analysis of the inhibition of the initial rate [^{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 significantly inhibited by any of the compounds.

Dixon plot analysis of SarCNU transport. In SK-MG-1 cells, the dixon plot analysis [128] of the effect of increasing concentrations of (±)-epinephrine and (±)-norepinephrine on the uptake of [^{3}H]SarCNU, at concentrations of 50 and 200 μM, at 37°C is shown in Figure 2. The influx of SarCNU was corrected for nonfacilitated diffusion in all cases [214]. The point of intersection of the two regression lines for (±)-epinephrine and (±)-norepinephrine is consistent with competitive inhibition of SarCNU influx by both compounds. The mean K_i for 3 independent experiments was found to be 140 ± 12 μM and 255 ± 41 μM for

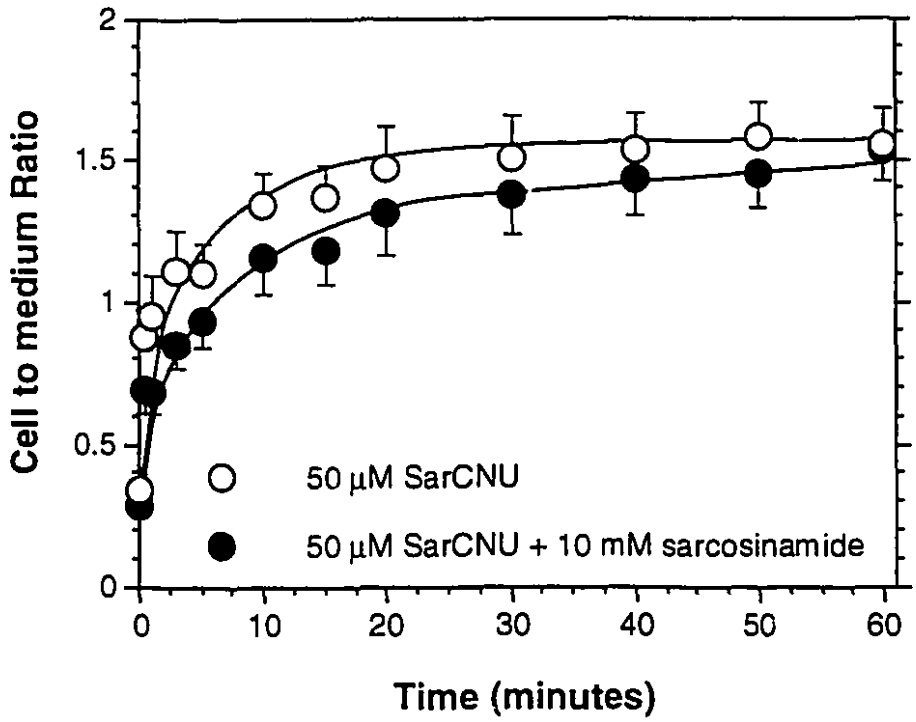


Figure 4.1. Comparison of the 60 minute time course of the influx of [^{3}H]SarCNU, at a 50 μM concentration, by SK-MG-1 cells in the presence and absence of 10 mM sarcosinamide at 37°C. The uptake, in suspension, is expressed as cell:medium distribution ratio, as described in "Materials and Methods." Points, mean of at least 6 separate experiments with each time interval performed in quadruplicate; bars, SE.

Table 4.1 Chemical specificity of the influx of SarCNU, at a concentration of 50 μM, in SK-MG-1 and SKI-1

SK-MG-1 and SKI-1 cells in suspension were examined at 37°C for 2 second uptakes of 50 μM [^{3}H]SarCNU in the absence or presence of the indicated inhibitors as described in "Materials and Methods."

Cell Line	Compound (10 mM)	n[a]	Initial Velocity[b] (pmol/ μl ICW/ sec)	% Control[c]	p[d]
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	N. S.[e]
	Sarcosinamide	8	2.85 ± 0.30	98 ± 10	N. S.
	Epinephrine	8	2.6 ± 0.3	88 ± 10	N. S.

[a] Number of individual experiments.

[b] Velocity values (average ± S.E.M.) were not corrected for diffusion.

[c] Percent difference (average ± S. E. M.) between control velocity versus velocity in the presence of inhibitor compound.

[d] Significance as determined by Scheffé test.

[e] Not significant

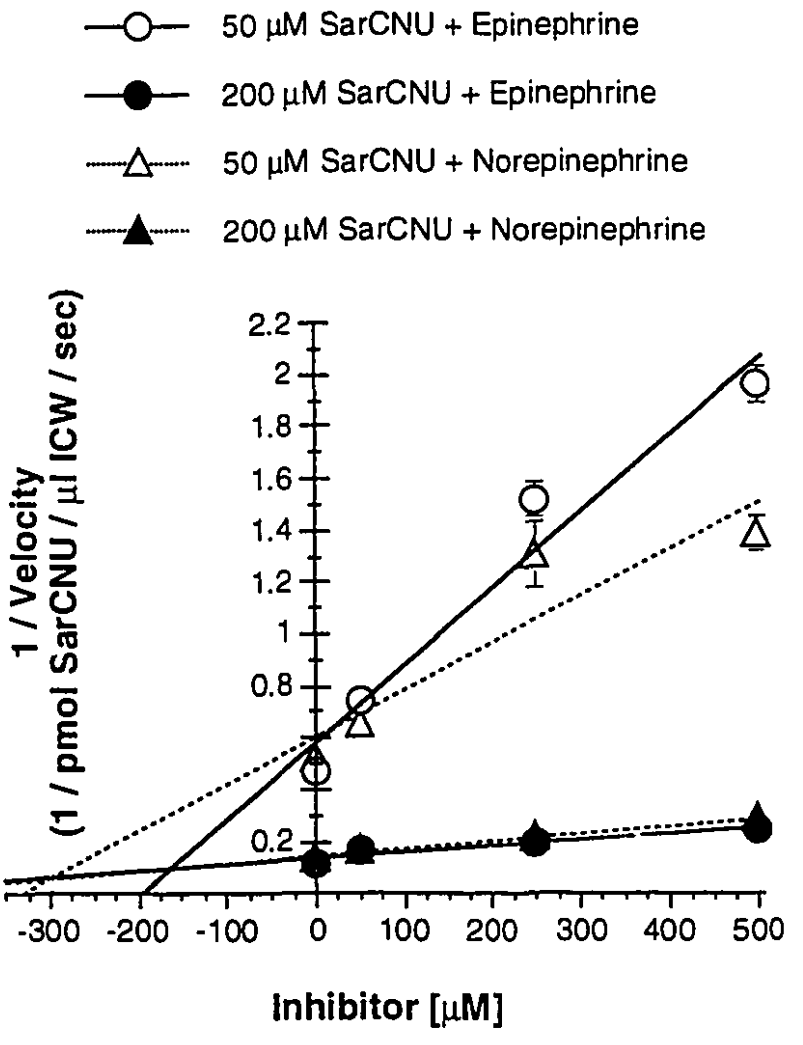


Figure 4.2. Dixon plot of (±)-epinephrine and (±)-norepinephrine inhibition of [^{3}H]SarCNU influx. The 2 second uptakes of [^{3}H]SarCNU, at concentrations of 50 and 200 µM, in media containing 50 to 500 µM (±)-epinephrine or (±)-norepinephrine were measured in suspension at 37°C, as described in "Materials and Methods." The K_i value for the average of all experimental values are 165 µM and 303 µM for (±)-epinephrine and (±)-norepinephrine, respectively. Points, mean of 3 separate experiments with each drug concentration performed in quadruplicate; bars, SE.

(±)-epinephrine and (±)-norepinephrine, respectively. The K_i value determined for (±)-epinephrine is similar to the K_i value determined previously [214].

Metabolism of [^{3}H](-)-norepinephrine. The intracellular metabolism of [^{3}H](-)-norepinephrine has to be determined since catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) are involved in the normal breakdown of norepinephrine [218]. 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 °C. The addition of up to 40 µM U-0521, a COMT inhibitor, for a 30 minute metabolism study demonstrated a recovery of 51% intact norepinephrine in SK-MG-1 versus 20% in untreated cells and 92% in all medias. The addition of up to 40 µM pargyline, a MAO inhibitor, did not increase the recovery of intact norepinephrine in SK-MG-1 cells. Furthermore, inhibition with both 10 µM U-0521 plus 10 µM pargyline did not significantly increase 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 [324, 521], the time course of metabolism of [^{3}H](-)-norepinephrine, at 25 µM and 10 mM concentrations without inhibitors, was performed at 37 °C. In the media, both drug concentrations had 84 ± 2% average intact

[^{3}H](-)-norepinephrine following the appropriate incubations. The recovery of intact intracellular [^{3}H](-)-norepinephrine at 0 seconds 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 seconds. 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 seconds, 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 seconds, respectively. Utilizing ANOVA analysis, there was no significant difference in the percent recovery of intact intracellular drug at 5 seconds 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°C.

Time course of [^{3}H](-)-norepinephrine influx at 0° and 37°C. 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 Figure 3. Following correction for metabolism, the influx of [^{3}H](-)-norepinephrine into both cell lines is linear to 15 seconds at 37°C. The initial rates of influx are similar for both cell lines at 37°C. In contrast, at 0°C, influx into SK-MG-1 cells is significantly inhibited compared to 37°C ($p = 0.009$), whereas, at 0°C, influx into SKI-1 cells is not significantly inhibited. Additionally, the initial rate of influx of [^{3}H](-)-norepinephrine at concentrations as high as 10 mM were found to be linear to 15 seconds (data not shown). All subsequent kinetic experiments were carried out at 5 seconds to approach the

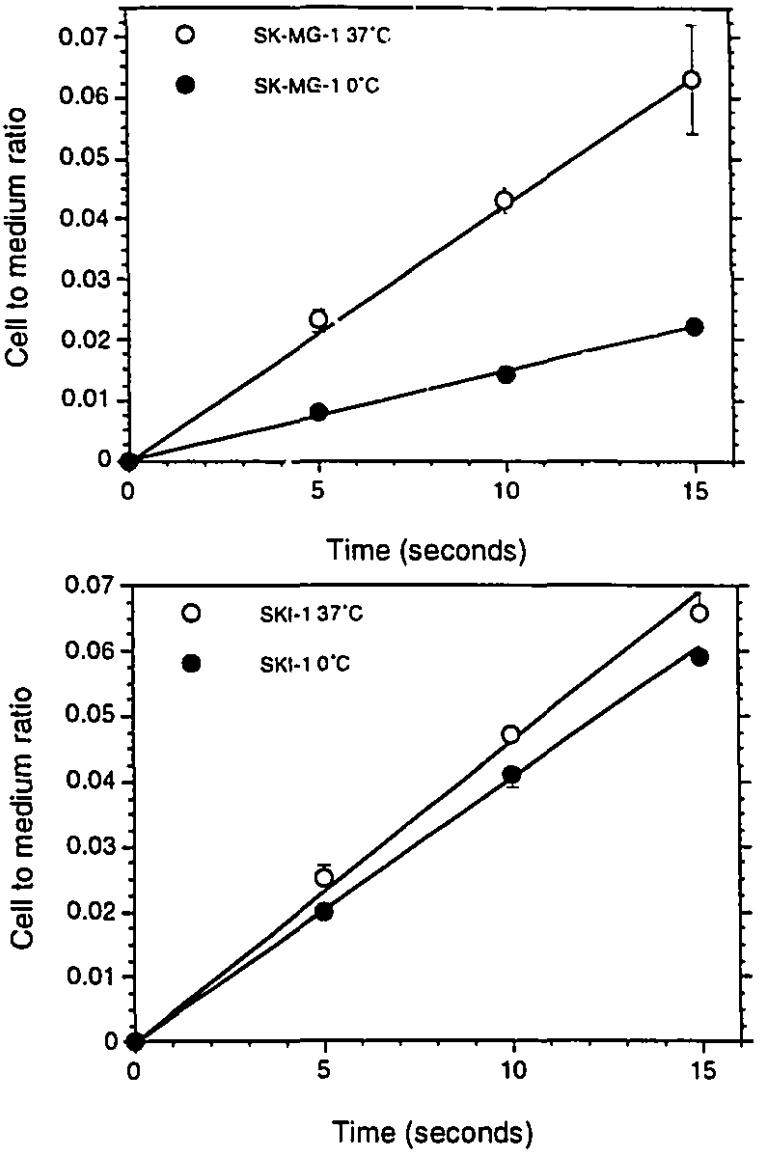


Figure 4.3. Time course of influx of [^{3}H](-)-norepinephrine, at a 25 µM concentration, by (A) SK-MG-1 cells and (B) SKI-1 cells in suspension at 0°C and 37°C. The uptake, in suspension, is expressed as cell:medium distribution ratio, as described in "Materials and Methods." The "zero-time" cell:medium ratios were equal to the background contributed by the contamination of the cell pellet with extracellular water space for 0 and 37°C. Points, mean of at least 4 separate experiments with each time interval performed in quadruplicate; bars, SE.

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 [123].

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 medium ratio, at 5 seconds, 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 cells versus concentration is shown in Figure 4 (Appendix 7 is a graphical representation of the equivalent relationship for SKI-1 cells). When concentrations of (-)-norepinephrine exceed 3 mM the velocity of influx into SK-MG-1 can be seen to be linearly dependent on concentration consistent with passive diffusion (Figure 4B). Regression of the velocities of influx between 3 and 10 mM allow for estimation of the nonfacilitated diffusion rate to be 1.2 ± 0.2 pmol/ µl ICW/ second/ 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 (Figure 4A). The corrected rate of (-)-norepinephrine influx into SK-MG-1 follows Michaelis-Menten kinetics, with non-linear regression analysis of the

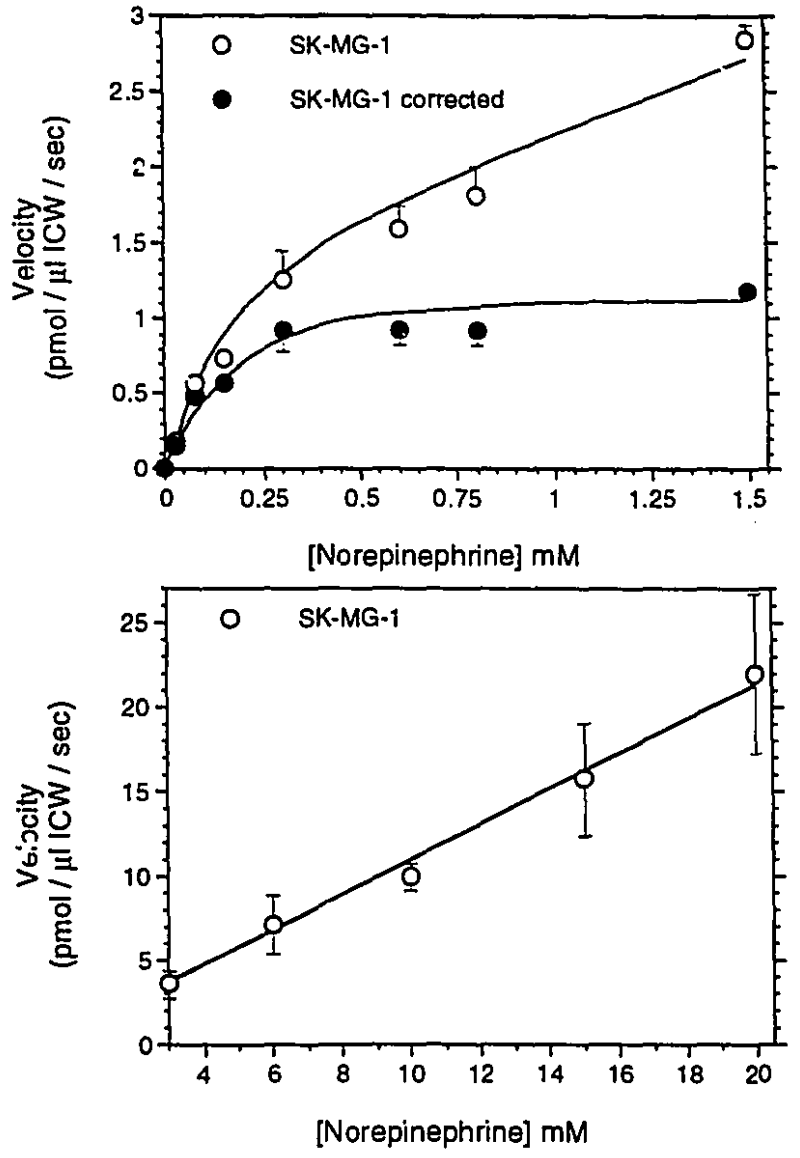


Figure 4.4. Influx of [^{3}H](-)-norepinephrine as a function of concentration. The 5 second uptakes of SarCNU from (A) 25 µM to 1.5 mM and (B) 3 mM to 10 mM were measured in suspension at 37°C, as described in "Materials and Methods." The corrected curve from (A) 25 µM to 1.5 mM represents the velocity minus the diffusional velocity of [^{3}H](-)-norepinephrine in the presence of excess (-)-norepinephrine. Points, mean of at least 4 separate experiments with each drug concentration performed in quadruplicate; bars, SE.

uncorrected data estimating a K_m of 148 ± 28 μM and a V_{max} of 1.23 ± 0.18 pmol/ μl ICW/ second.

In contrast to SK-MG-1 cells, SKI-1 cells do not demonstrate a decrease in the cell to medium ratio as [^{3}H](-)-norepinephrine is 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 is consistent with entry of (-)-norepinephrine into these cells via passive diffusion.

Dixon plot analysis of [^{3}H](-)-norepinephrine transport. In SK-MG-1 cells, the Dixon plot analysis [128] 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°C is shown in Figure 5. The influx of (-)-norepinephrine was corrected for non-facilitated diffusion in all cases. The point of intersection of the two regression lines for (-)-epinephrine (Figure 5A) and SarCNU (Figure 5B) is consistent with competitive inhibition of (-)-norepinephrine influx by both compounds. The mean K_i for 3 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 is similar to the K_i value determined previously in Dixon plot analysis of ^{3}[H]SarCNU uptake with epinephrine inhibition [214]. The K_i value determined for SarCNU is similar to the K_m value for SarCNU transport, determined previously [214].

Chemical specificity of [^{3}H](-)-norepinephrine and SarCNU transport. Neuronal uptake$_1$ and extraneuronal uptake$_2$ inhibitors were

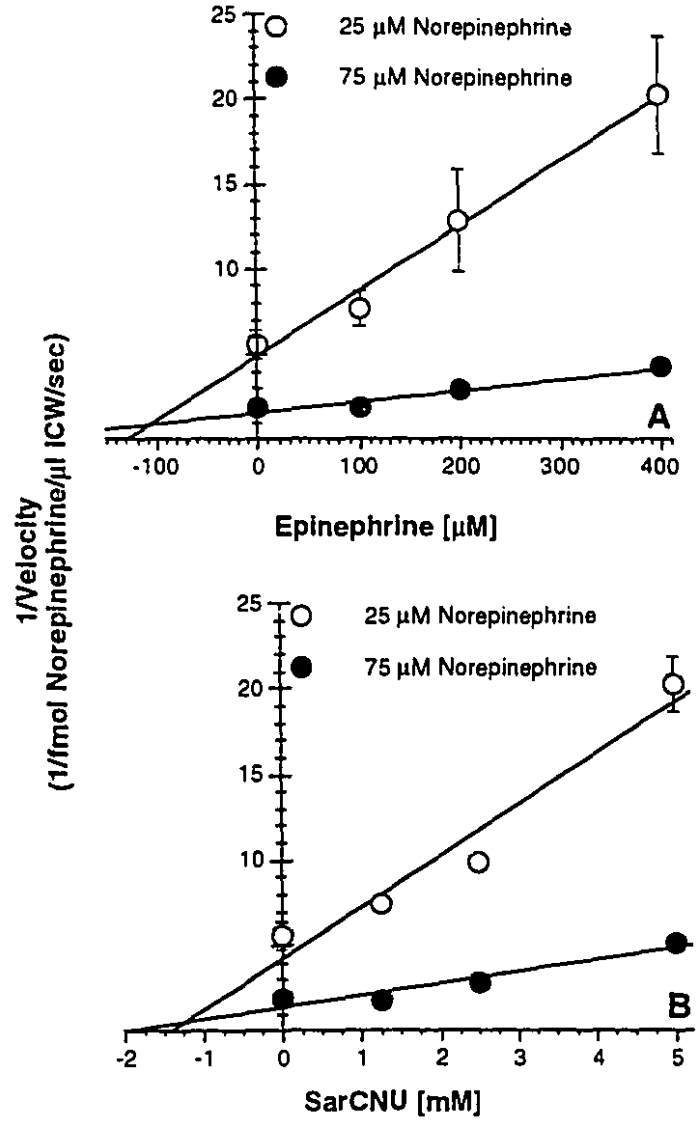


Figure 4.5. Dixon plot of (-)-epinephrine and SarCNU inhibition of [^{3}H](-)-norepinephrine influx. The 5 second uptakes of [^{3}H](-)-norepinephrine, at concentrations of 25 and 75 µM, in media containing (A) 50 to 500 µM (-)-epinephrine or (B) 1.25 to 5 mM SarCNU were measured in suspension at 37°C, as described in "Materials and Methods." The K_i value for the average of all experimental values are 104 µM and 1.2 mM for (-)-epinephrine and SarCNU, respectively. Points, mean of 3 separate experiments with each drug concentration performed in quadruplicate; bars, SE.

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 [108], and cimetidine, also an inhibitor of the renal cationic transporter [326]) 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[317]. The presence of the Na^+/K^+ ATPase inhibitor, ouabain, and excess external KCl are capable of depolarizing membrane potential [320]. Therefore, the initial rate of influx of [^{3}H](-)-norepinephrine, at a 25 μM concentration, into SK-MG-1 cells, at 37°C, was examined in the presence of 100 mM KCl, with an equimolar decrease in NaCl, or 0.4 mM ouabain. In 3 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 significantly affected by ouabain [214] or by 100 mM KCl (cell to medium ratio of 0.19 ± 0.01 and 0.20 ± 0.02 for control and 100 mM KCl, respectively.).

Table 4.2 Chemical specificity of the influx of Norepinephrine and SarCNU into SK-MG-1 cells

SK-MG-1 cells in suspension were examined at 37°C for 5 or 2 second uptakes of [^{3}H](-)-norepinephrine or [^{3}H]SarCNU, respectively, in the absence or presence of the indicated inhibitors as described in "Materials and Methods."

Compound	Inhibitor	n[a]	Initial velocity[b]	% Control[c]
25 µM Norepinephrine			fmol/ µl ICW/ sec	
	Control	3	285 ± 13	-
	10 mM Epinephrine	3	55 ± 17	19 ± 5[d]
	10 mM Sarcosinamide	3	33 ± 7	12 ± 2[d]
	100 µM Papaverine	3	75 ± 25	26 ± 9[d]
	1 µM Disprocynium-24	3	68 ± 26	23 ± 8[d]
	100 µM Cimetidine	3	118 ± 24	42 ± 10[d]
	10 µM Desipramine	3	287 ± 18	100 ± 5[e]
50 µM SarCNU			pmol/ µl ICW/ sec	
	Control	4	4.43 ± 0.03	-
	10 mM Epinephrine	4	2.34 ± 0.23	53 ± 6[d]
	10 mM Sarcosinamide	4	2.78 ± 0.03	63 ± <1[d]
	100 µM Papaverine	4	2.77 ± 0.22	59 ± 4[d]
	1 µM Disprocynium-24	4	2.28 ± 0.14	52 ± 4[d]
	100 µM Cimetidine	4	2.61 ± 0.38	64 ± 8[d]
	10 µM Desipramine	4	4.17 ± 0.29	93 ± 7[e]

[a] Number of individual experiments.

[b] Velocity values (average ± S. E. M.) were not corrected for diffusion.

[c] Percent difference (average ± S. E. M.) 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 Scheffé ANOVA test.

[e] Not significantly different from control velocity.

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 [214]. 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 competitively inhibit 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 [522]. 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 [108]. Sarcosinamide has also shown to reduce the *in vitro* toxicity of SarCNU against SK-MG-1 cells [500]. 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 minute 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 [213, 215] These results were obtained indirectly through the use of [^{3}H]SarCNU. In the present study, [^{3}H](-)-norepinephrine was used to directly address 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) [312, 314] The uptake$_2$ transporter is involved in an intracellular metabolizing cascade of norepinephrine and epinephrine via COMT and MAO [218]. Therefore, the ability of both SK-MG-1 and SKI-1 cells to metabolize norepinephrine was investigated. Following 30 minutes 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, a MAO inhibitor, did not effect the recovery of intact norepinephrine in either cell line. These results suggest that the primary method of inactivation of norepinephrine in both SK-MG-1 and SKI-1 is COMT, consistent with uptake$_2$ transport. Since U-0521, a COMT inhibitor, is known to inhibit uptake$_2$ transport [324, 521], 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 seconds in either cell line.

The influx of norepinephrine is linear to 15 seconds 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 is saturable in SK-MG-1 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 competitively inhibited 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 ß-haloalkylamines [515] 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 [326]. However, the catecholamine uptake$_2$ transporter has been described in only two cell lines [326, 327]. 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 [218]. Carrier mediated uptake of catecholamines has been demonstrated in rodent astrocytes [241, 305]. 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 previously been shown to inhibit influx, consistent with the observation that it shares a common carrier for catecholamines and SarCNU [108]. 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 are 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$_1$, was unable to significantly inhibit the influx of norepinephrine and SarCNU [523]. 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 [323], and the renal cationic inhibitor, cimetidine [326, 327], 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 [317, 321]. These results have lead to the suggestion that it is the positively charged substrate that is transported by the uptake$_2$ transporter [320]. In this study, membrane depolarization was achieved by excess extracellular KCl and the addition 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 significantly effected by either ouabain [214] 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 directly [226]. 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 ≤ 2.1 nM and 2.8 nM, respectively [228]. Furthermore, following stimulation, the release of *in vivo* norepinephrine at selected locus ceruleus target areas in the rat has resulted in an increase norepinephrine concentrations to a maximum of 2.4 μM [229]. Plasma concentrations of resting norepinephrine and epinephrine levels are 3 nM and 0.3 nM, respectively [230]. These relatively low concentrations of norepinephrine and epinephrine would not significantly interfer 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 [215]. 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 to BCNU in athymic mice bearing human gliomas indicates a superior therapeutic index for SarCNU [524].

CHAPTER 5

GENERAL DISCUSSION

Treatment of malignant human tumors of astrocytic origin is complicated by fact that 40 to 60% of these tumors demonstrate insensitivity to CENUs upon primary exposure and often acquire resistance during the course of therapy [98, 99]. The present investigation has concentrated on the examination of the mechanism of enhanced cytotoxicity of SarCNU in human glioma cell line SK-MG-1 versus resistant SKI-1. Emphasis was placed on the analysis of cellular transport of SarCNU since, unlike other CENUs, there have been indirect indications that SarCNU influx may be mediated by an epinephrine sensitive pathway [108]. The purpose of this discussion is to evaluate the preceding results with regard to the observation that SarCNU is more cytotoxic to primary human glioma cells, *in vitro* [104], and to a human glioma xenografted into athymic mice than clinically available BCNU [105]. Additionally, there will be an attempt to suggest possible reasons for the fact that SarCNU has been shown to cause decreased systemic cytotoxicity in mice [17, 105] and to be less cytotoxic to human bone marrow *in vitro* [105, 106].

5.1 Background

The analysis of CENU cytotoxicity in human glioma cell lines SK-MG-1 and SKI-1 has revealed that SKI-1 cells are 20 fold resistant to SarCNU and 3 fold resistant to BCNU in the HTSCA assay [104]. The ratio of SarCNU to BCNU dose required to kill 70% of cells in the HTSCA assay was 2 for SK-MG-1 cells and 12 for SKI-1 cells. The fact that SK-MG-1 cells are more sensitive to SarCNU relative to BCNU is suggested by a decreased SarCNU to BCNU effective dose ratio in SK-MG-1 cells compared to SKI-1 cells. The decreased effective dose ratio is also reflected by

the increased level of sensitivity to SarCNU compared to BCNU in SK-MG-1 cells versus SKI-1 cells. At the time, the reason for the increased effectiveness of SarCNU was not clear. The difference in sensitivity of SK-MG-1 and SKI-1 cells to either SarCNU or BCNU is not to be due to MGMT since both cell lines have been determined to be Mer^- [213]. The possibility that the influx of SarCNU into SK-MG-1 cells may be carrier-mediated was demonstrated by the fact that the presence of sarcosinamide, the carrier moiety of SarCNU, during cytotoxicity studies significantly reduced the cytotoxic effect of SarCNU but not BCNU [500], which enters cells via passive diffusion [109]. The effect of sarcosinamide on SarCNU cytotoxicity against SKI-1 cells was not analyzed. Interestingly, the steady state accumulation of SarCNU was demonstrated to be double that of BCNU in SK-MG-1 cells [108]. The likelihood that SarCNU accumulation into SK-MG-1 cells was mediated by a carrier-mediated system was further supported by the demonstration of the competitive inhibition of sarcosinamide influx by SarCNU [108]. The study also identified a mutual competitive inhibition of sarcosinamide and epinephrine for the a carrier-mediated system.

Comparison of sarcosinamide influx into SKI-1 cells revealed that the affinity of SKI-1 cellular uptake for sarcosinamide was reduced 5 fold in comparison to SK-MG-1 cells [213]. Furthermore, the steady state accumulation of SarCNU was approximately two fold greater in SK-MG-1 compared to SKI-1 cells whereas the accumulation of BCNU was comparable in both cell lines [108, 213]. The increased steady state of accumulation in SK-MG-1 cells paralleled the approximate 41% increase in area under the curve of DNA ISCLs compared to SKI-1 cells. The reduction of ISCL in SKI-1 cells was not associated with any significant

alteration in DNA repair or detoxification pathways for CENUs [213]. From these results, it was suggested that the resistance of SKI-1 cells to SarCNU was associated with a decreased intracellular accumulation of SarCNU, potentially associated with altered transport, with concomitant reduction in ISCL formation.

5.2 Transport of SarCNU into SK-MG-1 cells

An important question in this thesis pertains to the identification of the nature of the potential carrier-mediated uptake of SarCNU into human glioma cells. The enhanced cytotoxicity of SarCNU may be a consequence of the increased chemical half-life of SarCNU versus BCNU [17] since it has been demonstrated that BCNU which possesses a short chemical half-life is rapidly degraded *in vivo* [7]. However, evidence suggests that the addition of sarcosinamide, an amino acid amide, to the N-3 portion of a CENU has generated a CENU which may be able enter cells by a method other than passive diffusion. The identification of a carrier for SarCNU may explain its increased cytotoxicity in SK-MG-1 cells and also in primary human gliomas relative BCNU. The intent of Chapter 2 was to directly address if SarCNU was transported into SarCNU sensitive SK-MG-1 cells.

In order to address the transport potential of SarCNU, tritium radiolabeled SarCNU was obtained and utilized for the direct analysis of influx. It was found that in the presence of excess unlabeled SarCNU the initial rate of influx of SarCNU was significantly reduced in SK-MG-1 cells. Furthermore, the initial rate of entry of SarCNU into SK-MG-1 cells was not reduced by Na^+ depletion or by addition of metabolic inhibitors. The influx of SarCNU into SK-MG-1 cells was demonstrated to

occur through both non-facilitated and facilitated diffusion. The definition of a transport system as facilitated diffusion requires that the mode of transport be energy independent, non-concentrative and saturable with demonstrable Michaelis-Menten kinetics [112]. Following correction for diffusion of the initial rate of influx of SarCNU into SK-MG-1 cells it was demonstrated that the K_m value obtained was similar to the K_i for inhibition by SarCNU of sarcosinamide influx (K_m of 2.39 mM versus K_i of 3.26 mM) [108]. The estimate of the K_i for SarCNU's inhibition of sarcosinamide influx did not correct for the rate of diffusion of sarcosinamide, which would have further reduced the estimate of K_i. K_i represents the concentration of inhibitor that causes a 50% reduction of total transport/influx. Correction for diffusion reduces the non-inhibitable portion of the estimate of influx and therefore increases the portion of specific carrier-mediated transport that contributes to total influx. The increased estimate of specific carrier-mediated transport contribution to total influx reduces the need to add more inhibitor necessary to inhibit total influx (specific transport plus diffusional influx and other potential carrier-mediated processes), therefore reducing the estimate of K_i.

Comparison of experimental versus theoretical values of the velocity of SarCNU influx at different substrate concentrations revealed that experimental values were 107 ± 16% of theoretical. The closeness of experimental to theoretical velocity values validates the kinetic constants obtained during experimentation. Therefore, at 67 μM, the theoretical PPC of SarCNU [104], facilitated diffusion can be calculated to account for 61% of total SarCNU influx into SK-MG-1 cells. (±)-Epinephrine was demonstrated to be able to completely inhibit the

facilitated diffusional contribution to the total influx of SarCNU into SK-MG-1 cells. The inhibition by (±)-epinephrine for specific carrier-mediated influx was demonstrated to be competitive. The value of K_i obtained for (±)-epinephrine was within the range of affinities for the transport of physiological substrates of the catecholamine uptake$_2$ transporter suggesting that SarCNU influx may be mediated through this transport system albeit at a greatly reduced affinity as compared to catecholamines [218, 326, 327]. The accumulation of SarCNU did not proceed against a concentration gradient nor was it inhibited by metabolic inhibition suggesting that SarCNU influx does not proceed via the neuronal uptake$_1$ of catecholamines [217]. Active transport of the alkylating agents melphalan through amino acid transport systems L and ASC [137, 138] and nitrogen mustard through a subtype of the choline transporter [353] has been demonstrated to proceed against a concentration gradient.

5.3 Relation of cytotoxicity to transport of SarCNU into SK-MG-1 and SKI-1 cells

Measurement of the steady state intracellular concentration of SarCNU via an insensitive colorimetric assay demonstrated increased SarCNU accumulation in SK-MG-1 cells versus SKI-1 cells [213]. In Chapter 3, the investigation of the difference in SarCNU accumulation and sensitivity between SK-MG-1 cells and SKI-1 cells was investigated. The clinically relevant PPC of 67 μM SarCNU has been demonstrated to reduce the viability of SK-MG-1 cells to greater than 70% of untreated cells but not SKI-1 cells. Analysis of primary tumors has demonstrated that anticancer agents that are effective at reducing the growth of

greater than 70% of tumor colonies in the HTSCA result in complete or partial responses approximately 60% of patients [525]. Taking this into consideration, SarCNU can be considered to be effective against SK-MG-1 cells but not SKI-1 cells.

Analysis of the steady state concentration of radiolabeled SarCNU, at a concentration similar to the theoretical PPC, in both cell lines revealed that SK-MG-1 cells accumulated 50% more SarCNU intracellularly than SKI-1 cells at 37°C. This is consistent with measurement of steady state levels of SarCNU utilizing mM concentrations of unlabeled SarCNU [213]. At 22°C, accumulation of SarCNU was found to be similar. The altered intracellular accumulation of SarCNU may be due to the greater initial rate of SarCNU influx into SK-MG-1 cells than SKI-1 cells at 37°C. This is supported by the similarity of accumulation and the lack of difference between the initial rate of influx at 22°C. Furthermore, altered accumulation was demonstrated not to be due to altered efflux or metabolism of SarCNU in either cell line. The existence of distinct influx and efflux routes for alkylating agent melphalan has been suggested [365].

The reduction of the intial rate of SarCNU influx into SKI-1 cells is consistent with the observation that influx of SarCNU into SKI-1 cells is technically non-saturable. The fact that SKI-1 cells possess no demonstrable transport system is in opposition to previous observations that SarCNU can competitively inhibit the influx of sarcosinamide into SKI-1 cells [213]. However, the K_i of SarCNU inhibition of sarcosinamide influx was found to be 17.5 mM which required concentrations of greater than 10 mM SarCNU during the course of experimentation. Analysis of the effect of 10 mM and greater

concentrations of SarCNU revealed inconsistent alterations in intracellular water which prevented utilization of sufficiently high concentrations required to demonstrate the presence or absence of a saturable carrier with a K_m of 17.5 mM.

In order to address the effect of altered intracellular SarCNU accumulation on cytotoxicity, monolayer cytotoxicity assays were performed. It was demonstrated that at 37°C SKI-1 cells were 3 fold resistant to SarCNU compared to SK-MG-1 cells. However, at 22°C the level of difference in sensitivity was reduced 2 fold. This significant alteration in the level of sensitivity to SarCNU may be simply due to a reduction in the temperature during the incubation period with the drug. However, sensitivity to BCNU, which enters cells purely by passive diffusion [109], was not significantly affected by alteration of the incubation temperature. The 2 fold level of resistance to SarCNU at 37°C is similar to the approximate 2 fold level of resistance of SKI-1 cells versus SK-MG-1 cells to BCNU at 22 and 37°C. The residual resistance to BCNU and SarCNU is most likely due to other mechanisms of resistance to CENUs but is not associated with any significant alteration in known DNA repair or detoxification pathways for CENUs [108, 213]. Interestingly, in SK-MG-1 cells there is a 55% reduction in the SarCNU to BCNU effective dose ratio at the IC_{90} when the temperature of incubation is increased from 22 to 37°C, whereas there is only a 30% reduction in the ratio when the temperature shift is examined in SKI-1 cells. The greater reduction of the SarCNU to BCNU effective dose ratio is a reflection of the increased cytotoxic effect that SarCNU has in SK-MG-1 cells at 37°C. The greater sensitivity of SK-MG-1 cells is also reflected by an increase in the IC_{90} concentration ratio required at

22°C versus 37°C for SarCNU which is 3.9 for SK-MG-1 cells but only 2.1 for SKI-1 cells with the ratios for BCNU being 1.8 for SK-MG-1 cells and 1.4 for SKI-1 cells. These data suggest that, at 37°C, when SK-MG-1 cells have a demonstrable epinephrine-sensitive SarCNU carrier-mediated uptake and SKI-1 cells do not, the level of sensitivity of SK-MG-1 cells compared to SKI-1 cells is increased. At 22°C, when there is no demonstration of a saturable carrier system in either cell line, the level of resistance is comparable to the innate resistance to CENUs that SKI-1 cells possess compared to SK-MG-1 cells. There are several examples of increased resistance related to decreased cellular uptake either by inhibition or reduced transport of the alkylating agents nitrogen mustard [357, 358, 360], melphalan [139, 368] and cis-platinum [403, 404]. However, to date, increased sensitivity related to alterations in accumulation of CENUs has not been demonstrated.

5.4 Characterization of the catecholamine extraneuronal uptake$_2$ status of SK-MG-1 cells and SKI-1 cells

As determined in Chapter 2, the uptake of SarCNU into SK-MG-1 cells occurs through an epinephrine-sensitive transporter whose affinity for (±)-epinephrine suggests the system is the catecholamine extraneuronal uptake$_2$ carrier. The lack of identification of such a SarCNU carrier-mediated influx into SKI-1 cells may be due to either lack of a transport system in SKI-1 or the fact that concentrations of SarCNU necessary to characterize a transporter could not technically be obtained. Sarcosinamide shares a transporter with epinephrine in SK-MG-1 cells which is unconfirmed in SKI-1 cells which have a carrier for sarcosinamide that has significantly reduced affinity for sarcosinamide

compared to SK-MG-1 cells [108, 213]. Therefore, the sarcosinamide transporter in SKI-1 cells may be different than that in SK-MG-1 cells. In order to confirm the identity of the transporter in SK-MG-1 cells and to address the presence or absence of a transporter in SKI-1 cells, radiolabeled (-)-norepinephrine was obtained for analysis of cellular uptake. The utilization of (-)-norepinephrine which has a μM affinity for the catecholamine uptake$_2$ transporter should be able to determine the presence or absence of a physiologically relevant transporter in either cell line. Chapter 4 deals with the characterization of norepinephrine transport in both cell lines.

Initially, SarCNU influx was analyzed to assess if sarcosinamide and epinephrine were able to inhibit influx into either cell line. There was no inhibition of SarCNU influx into SKI-1 cells by excess concentrations of SarCNU, sarcosinamide or epinephrine. This strongly suggests that in SKI-1 cells SarCNU is (a) not transported by a carrier mediated mechanism and (b) that it does not share a carrier with either sarcosinamide or epinephrine. However, significant and equivalent inhibition of SarCNU influx into SK-MG-1 cells by sarcosinamide and epinephrine suggests that all three compounds share a common transporter. (-)-Norepinephrine and (-)-epinephrine were demonstrated to competitively inhibit SarCNU influx into SK-MG-1 with affinities consistent with those previously identified for the extraneuronal uptake$_2$ transporter [218, 522]. Considering the potential identity of the SarCNU transporter it is interesting to note that inhibition of SarCNU influx into SK-MG-1 cells with sarcosinamide over a one hour period significantly reduced intracellular accumulation of SarCNU. More importantly, this suggests that reduced toxicity of SarCNU in the

presence of sarcosinamide [500] is a consequence of reduced accumulation mediated through inhibition of the SarCNU/sarcosinamide transporter. The reduction of cytotoxicity is not due to non-specific alteration of CENU activity by sarcosinamide since sarcosinamide did not alter BCNU cytotoxicity [500].

Analysis of (-)-norepinephrine influx demonstrated that influx was temperature dependent into SK-MG-1 cells but not SKI-1 cells. The influx of (-)-norepinephrine into SK-MG-1 was demonstrated to occur through both non-facilitated and facilitated diffusion with influx occurring exclusively by passive diffusion in SKI-1 cells. Following correction of the initial rate of influx of (-)-norepinephrine into SK-MG-1 cells for diffusion it was demonstrated that the K_m value obtained was similar to the K_i for inhibition by (±)-norepinephrine of SarCNU influx (K_m of 148 μM versus K_i of 255 μM). Comparison of the experimental versus theoretical values of the velocity of (-)-norepinephrine influx at different substrate concentrations revealed that experimental values were 99 ± 8% of theoretical values. The closeness of experimental to theoretical velocity values validates the kinetic constants obtained during experimentation.

At 2.4 μM (-)-norepinephrine, the maximum level of (-)-norepinephrine detected at a rat axon [229], facilitated diffusion would account for 87% of total (-)-norepinephrine influx into SK-MG-1 cells. Both (-)-epinephrine and SarCNU are capable of competitively inhibiting the carrier-mediated influx into SK-MG-1 cells. The K_i for (-)-epinephrine is similar to the K_i for (±)-epinephrine for inhibition of SarCNU influx (K_i of 111 μM versus 140 μM). Furthermore, the K_i for SarCNU inhibition of (-)-norepinephrine influx is similar to the K_m for

SarCNU influx into SK-MG-1 cells (K_i of 1.48 mM versus 2.39 mM). These results suggest that in SK-MG-1 cells the transport of SarCNU, epinephrine and norepinephrine is through a common carrier.

The fact that epinephrine has a greater affinity than norepinephrine for the shared SarCNU transporter suggests that the transport system is the catecholamine extraneuronal uptake$_2$ transporter since this relationship is firmly established for the transporter [221, 246, 306]. The characterization of the chemical specificity of both SarCNU and (-)-norepinephrine influx demonstrated that the transport of both compounds into SK-MG-1 cells occurs via the extraneuronal uptake$_2$ transporter. However, the uptake of SarCNU was not inhibited by depolarization which would suggest that unlike catecholamines the SarCNU species transported by uptake$_2$ is not positively charged. This prediction has been strengthened by the observation that the dichloromethane/water partition coefficient of SarCNU does not vary with pH (pH 1.4 to 9.0) which indicates that SarCNU is not ionized over the pH range tested [18].

5.5 Concluding remarks

Saturable transport of a non-selective uptake$_2$ substrate has been demonstrated in several primary human gliomas but not characterized directly with catecholamines [226]. We have characterized the extraneuronal uptake$_2$ of (-)-norepinephrine in SK-MG-1 cells. This is the first detailed description of the uptake$_2$ system of transport in human glioma cell lines. Furthermore, we have demonstrated that the uptake$_2$ system can mediate the transport of an apparently neutral SarCNU molecule and that the catecholamine neuronal uptake$_1$ transporter is not

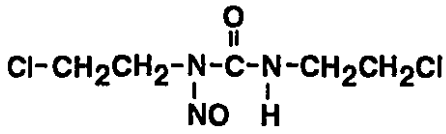
involved. The compiled results strongly suggest, as tested through the classic ABC test for shared uptake at a carrier-site [119, 120], that transport of SarCNU, epinephrine and norepinephrine occurs via the same transporter. The ABC test requires that the K_i of compound A inhibiting the uptake of compound B be essentially the same as the K_m of compound A. An additional test for a shared transporter has been suggested since there may be variation of these parameters in experimentation [118, 197]. This test requires that a dissimilar analog(s) (compound C), presumably transported by the same carrier-site, show the same K_i value as an inhibitor of influx of both compounds A and B. In the case of SK-MG-1 cells the ABC test validates the uptake$_2$ transporter as that which mediates the uptake of SarCNU, epinephrine and norepinephrine since: test AB; the K_i for SarCNU inhibiting (-)-norepinephrine uptake (K_i of 1.48 mM) is similar in value to the K_m of SarCNU (K_m of 2.39 mM)(p = 0.0996) and test C; the K_i for (±)-epinephrine inhibiting SarCNU uptake (K_i of 140 μM) is similar to the K_i for (-)-epinephrine inhibiting (-)norepinephrine uptake (K_i of 111 ± 6 μM)(p = 0.1337). An incomplete ABC test also suggests that sarcosinamide, the carrier moiety of SarCNU, can also be transported by the uptake$_2$ transporter, since in SK-MG-1 cells: (a) the K_i for (±)-epinephrine inhibiting sarcosinamide uptake is similar to the K_m of (±)-epinephrine [108] and (b) the K_i for SarCNU inhibiting sarcosinamide uptake is similar to the K_m of SarCNU. In addition, sarcosinamide inhibited the influx of SarCNU and (-)-norepinephrine at a level similar to that predicted by calculation utilizing experimentally obtained kinetic parameters (theoretical inhibition of SarCNU and (-)-norepinephrine of 59% versus actual at 37% and 80% versus actual 88%, respectively). These results also suggest

that transport of catecholamines via uptake$_2$ in SK-MG-1 cells is not stereochemically selective, since affinities (K_m or K_i) of the (±)-catecholamine are similar to (-)-catecholamine. The structural similarities between SarCNU, sarcosinamide, epinephrine and norepinephrine that may account for their mutual recognition by the uptake$_2$ transporter appear to be the tertiary nitrogen (N-methyl group) and the separation of two nitrogen atoms by a carbon atom (see Figure 5.1). However, this observation remains to be validated.

The highest level of norepinephrine detected at the neuronal junction in rat is 2.4 μM [229]. Assuming that the maximum concentration of norepinephrine is similar at a human neuronal junction, the theoretical peak plasma concentration (PPC) of SarCNU would inhibit carrier-mediated norepinephrine influx by only 2.7% and total influx by only 2.3% (ac 2.4 μM norepinephrine facilitated diffusion accounts for approximately 87.2% of total influx with passive diffusion accounting for only 12.8%). In terms of the inhibition of the influx of SarCNU at its theoretical PPC, 2.4 μM norepinephrine would inhibit carrier-mediated SarCNU influx by only 1.6% and total influx by only 1% (at 67 μM SarCNU (PPC) facilitated diffusion accounts for approximately 61% of total influx with passive diffusion accounting for only 39%). The level of inhibition by 2.4 μM norepinephrine is similar for concentrations of SarCNU as low as 1 μM. At the theoretical PPC of SarCNU the velocity of uptake is 27% of its maximal velocity for carrier-mediated influx which is greater than the velocity of norepinephrine at 2.4 μM which would account for 1.6% of its maximal velocity. At 1-9 μM SarCNU, a concentration attainable with clinically utilized CENUs, approximately 60% of total influx would be via the facilitated diffusion which

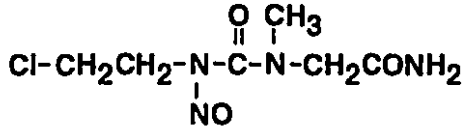
BCNU

1, 3-bis-(2-chloroethyl)-1-nitrosourea



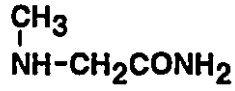
SarCNU

(2-chloroethyl)-3-sarcosinamide-1-nitrosourea

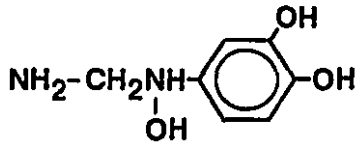


Sarcosinamide

***N*-methylglycinamide**



Norepinephrine



Epinephrine

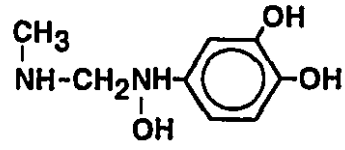


Figure 5.1 Structures of BCNU, SarCNU, sarcosinamide, epinephrine and norepinephrine.

represents an important contribution to SarCNU uptake. Velocity for carrier-mediated influx would account for 3.8% and 0.4% of maximal carrier-mediated influx at 9 μM and 1 μM SarCNU, respectively. It therefore seems that physiologically relevant concentrations of norepinephrine will not significantly affect the intracellular accumulation of SarCNU via the catecholamine uptake$_2$ transporter. Moreover, even if the SarCNU concentration reaches its theoretical PPC within the brain, it should not significantly interfer with the normal catecholamine signaling process with regards to inhibition of reuptake via uptake$_1$ into the neuron and uptake$_2$ into glial cells. Therefore, it is probable that carrier-mediated uptake via the catecholamine uptake$_2$ transporter contributes to the enhanced cytotoxicity of SarCNU as compared to BCNU, which enters cells via passive diffusion [109], in primary human glioma cells [104].

Malignant human gliomas are characterized by their significant heterogeneity [526]. In fact, two Mer$^-$ glioma cell lines obtained from the same tumor specimen of a patient with glioblastoma have been demonstrated to vary markedly in sensitivity to irradiation and BCNU [527]. It therefore comes as no surprise that the Mer$^-$ glioma cell lines SK-MG-1 and SKI-1 differ in their sensitivity to BCNU. Sensitivity to SarCNU is altered at an additional level, due to altered transport, when comparing both cell lines. As discussed, SK-MG-1 cells possess the extraneuronal uptake$_2$ transporter which mediates the carrier-mediated influx of SarCNU but SKI-1 cells appear not to have a physiologically relevant transporter of SarCNU. Indeed, even if SKI-1 cells do have a unique transporter for SarCNU its lack of affinity and/or turnover rate for SarCNU is not able to overcome the tremendous

diffusion potential of SarCNU into SKI-1 cells (the estimated diffusion potential is 108 ± 13 pmol/µl ICW/sec/mM SarCNU). Interestingly, the activity of the extraneuronal O-methylating system appears to be regulated by adrenergic innervation. It has been demonstrated that sympathetic denervation of tissues leads to reduced O-methylation of isoprenaline which was associated with distinct morphological changes of smooth muscle characterized as cellular de-differentiation [528]. By extension, establishment of human glioma cell lines, by definition, removes adrenergic innervation which may or may not lead to loss of the extraneuronal O-methylating system including the uptake$_2$ transporter. Alternatively, there might be subpopulations of human glial cells that either express the uptake$_2$ transport system or not. The lack of the uptake$_2$ transporter in SKI-1 cells may be a consequence of these reasons or SKI-1 cells may have lost the system through establishment as a cell line.

Reduced systemic and bone marrow toxicity of SarCNU may be purely a consequence of its increased chemical half-life versus classical CENUs such as BCNU. In fact, it has been demonstrated that reduced chemical half-life of CENUs correlates with decreased general cytotoxicity associated with CENU treatment [36]. It is possible that specific uptake into tissues via the extraneuronal uptake system could alter the general toxicity of SarCNU. Thus, further analysis would be required to see if SarCNU is more toxic to tissues known to contain uptake$_2$ transport.

The presence of the SarCNU/catecholamine uptake$_2$ transporter in SK-MG-1 cells has been correlated with a 54% increase in intracellular SarCNU accumulation compared to SKI-1 cells [213]. This evidence

strongly suggests that the increased accumulation accounts for the enhanced level of sensitivity of SK-MG-1 cells to SarCNU. In fact, the increased steady state of accumulation in SK-MG-1 cells parallels the approximate 41% increase in DNA ISCLs compared to SKI-1 cells (29% decrease) [213]. The increase in ISCL in SK-MG-1 cells was not associated with any significant alteration in DNA repair or detoxification pathways for CENUs compared to SKI-1 cells. The increase in the number of ISCLs and intracellular accumulation following SarCNU exposure are consistent with increased cytotoxicity in SK-MG-1 cells versus SKI-1 cells. Few attempts have been made to correlate the formation of DNA cross-links with cytotoxicity of alkylating/platinating agents. However, in the case of human glioma cell lines, a significant difference in cell survival (100 to 1000 fold) was accompanied by a 2 fold decrease in formation of DNA ISCLs within a cell line treated with different concentrations of ACNU [50]. This suggests that there may be an exponential relationship between chemosensitivity and the DNA cross-link factor within a particular cell line. Interestingly, in comparing sensitive and resistant cells a 6.5 fold ACNU resistant cell line accumulates 3.5 times fewer DNA ISCLs versus a sensitive cell line. In rat glioma cell lines a 2 fold decrease in ISCLs has been associated with only a 16 fold level of ACNU resistance [466]. A 4 fold reduction in the accumulation of melphalan has also been associated with a 30 fold resistant human breast cancer cell line [139]. This reduction in accumulation was demonstrated to be associated with an alteration of the kinetic parameters of the amino acid transport system L. In the case of murine leukemia L1210 cells with 20 to 110 fold resistance to cisplatinum, a 40% reduction in accumulation was associated with a 20 to

36% reduction in total DNA platination [513]. The significance of the reduction of cisplatin accumulation is not clear since the resistant cell lines also demonstrated increased repair of DNA-platinum adducts. Other studies involving cisplatinum and its derivatives have also associated reduced accumulation of platinating agent with resistance. However, these studies did not directly address the level of DNA platination associated with altered accumulation. The aforementioned studies have analyzed resistance mechanisms to cytotoxic compounds which involved the stepwise exposure of a sensitive wild type cells to increasing drug concentrations. This selection has been suggested to result in more than one phenotypic trait involved in resistance to a particular cytotoxic drug, particularly if the stepwise selection occurs over an extended period of time [406]. Therefore, it remains to be determined if altered DNA cross-linking is as a consequence of enhanced DNA repair, increased drug detoxification or reduced cellular accumulation. In fact, enhanced resistance to cross-link induction may be a consequence of alterations in many pathways associated with resistance to a particular chemotherapeutic compound.

In summary, the results of this analysis of SarCNU and norepinephrine influx into human glioma cell lines SK-MG-1 and SKI-1 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. 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 to BCNU in athymic mice bearing human gliomas indicates a superior therapeutic index for SarCNU [524].

Appendix 1 Trans-stimulation of SarCNU influx

Materials and Methods

Measurement of trans-stimulation of [^{3}H]SarCNU uptake. Trans-stimulation experiments were performed by a modification of a previously described technique [510]. Briefly, cells in suspension ($4x10^6$ cells/ml), at 22°C, were preincubated for 15 minutes. Following the pre-incubation, cells were diluted 2-fold with either PAG or osmotically adjusted PAG containing 20 mM SarCNU pH 7.4 (final concentration 10 mM) and incubated for 20 minutes. The loaded cells were aliquoted (400 µl) into 1.5 ml microcentrifuge tubes and centrifuged for 5 minutes at 300 x g. Medium was aspirated, leaving approximately 10 µl of loading media and cell pellet left at 22°C until used for trans-stimulation. Immediately before use, each cell pellet was gently disrupted. To initiate analysis of uptake of SarCNU, the cell suspension was resuspended in 400 µl of 10 µM [^{3}H]SarCNU (specific activity of 34.2 µCi/µmol). The final external concentration of SarCNU for the preloaded cells is approximately 500 µM versus 10 µM in the control cells. At 3 seconds, the earliest obtainable time point with this technique, transport was terminated by separating the cells from the media by centrifugation through versilube oil, as described above. An aliquot of the medium was removed and retained for analysis of [^{3}H]SarCNU content. The cell pellet was processed as described in Chapter 2, "Materials and Methods" . Every time point was performed in quadruplicate.

In order to perform the analysis of membrane associated SarCNU, approximately 25 µl of media was left following aspiration of loading

media. Analysis of membrane associated SarCNU radioactivity was performed by rapid addition of the concentrated cell suspension to 400 µl of 50 µM [^{3}H]SarCNU (specific activity of 6.84 µCi/µmol) resting on 1.0 ml of Versilube F-50 silicone oil followed by immediate centrifugation. This zero-time, membrane associated radioactivity was subtracted from every point.

Results

Trans-stimulation of [^{3}H]SarCNU Uptake in SK-MG-1. The effect of intracellular SarCNU on the initial uptake of [^{3}H]SarCNU, in SK-MG-1, was examined by preloading cells with either PAG or PAG with 10 mM SarCNU and then comparing the uptake of 10 µM [^{3}H]SarCNU at 37°C between both sets of cells. The analysis was performed under initial uptake conditions at 3 seconds as a consequence of experimental restrictions. The preloading procedure of SK-MG-1 cells causes the external concentration of [^{3}H]SarCNU to be approximately 500 µM compared to 10 µM for control cells. Although this is a difference of 50 fold in concentration the preloading of SK-MG-1 with excess of unlabeled SarCNU increased the accumulation of SarCNU to 133 ± 8% of control unloaded cells. This difference is statistically significant ($p < 0.01$) and suggests the involvement of a carrier for the uptake of SarCNU in SK-MG-1 cells.

Discussion

The uptake of SarCNU into SK-MG-1 cells was found to be trans-stimulated by excess intracellular SarCNU. This is technically not a trans-stimulation experiment since the external concentrations of SarCNU

differ between the preloaded and control cells. However, when the derived kinetic constants for K_m, V_{max} and the diffusion rate constant of SarCNU in SK-MG-1 cells are utilized to solve the Michaelis-Menten equation in "Materials and Methods" a different result is expected. The preloaded cells are expected to show approximately 90% of the accumulation noted in the control cells. Yet, there is a significant increase in SarCNU accumulation suggesting that a form of trans-acceleration has occurred in the preloaded cells. Trans-stimulation or acceleration of the influx of SarCNU suggests that its uptake is dependent, partially, on a reversible, carrier-mediated transport system [133].

Appendix 2 Inhibition of SarCNU influx with amino acids

Table A.2.1 Inhibition studies with [^{3}H]SarCNU, at a 50 μM concentration, in SK-MG-1 cells

SK-MG-1 cells in suspension were examined at 37°C for 2 second uptakes of[^{3}H]SarCNU, at a concentration of 50 μM, in the absence or presence of the indicated inhibitors as described in "Materials and Methods." of Chapter 2.

SarCNU	Concentration (mM)	% of control uptake[a,b]
SarCNU	10	76 ± 5[c]
Lysine	10	111 ± 19[d]
Threonine	10	112 ± 11[d]
Leucine	10	122 ± 13[d]
Glycine	10	108 ± 8[d]

[a] The velocity of uptake of 50μM SarCNU was 6.25 ± 0.50 pmol SarCNU/μl ICW/second. Velocity values (average ± S.E.M.) were not corrected for diffusion.

[b] Percent difference (average ± S. E. M.) between control velocity versus velocity in the presence of the compound tested for inhibition.

[c] Difference was determined to be significant, $p < 0.05$, by the two-tailed t test.

[d] Differences were determined to be not significant by the two-tailed t test.

Appendix 3 Metabolic inhibition of SarCNU influx

Table A.3.1 Effect of metabolic inhibitors on the transport of [^{3}H]SarCNU in SK-MG-1 cells

SK-MG-1 cells were treated with the indicated inhibitor for 15 minutes, at 37°C, prior to examining uptake of 50 μM [^{3}H]SarCNU at 2 seconds. The values represent the mean ± SE of four separate experiments.

Compound	Concentration (mM)	% of control uptake[a,b]
2,4-Dinitrophenol	0.4	119 ± 18[c]
Na.	2.0	123 ± 11[c]
Iodoacetate	2.0	118 ± 9[c]
Ouabain	0.4	96 ± 11[c]
NaCN	1.0	112 ± 10[c]

[a] The velocity of uptake of 50μM SarCNU was 4.75 ± 0.75 pmol SarCNU/μl ICW/second. Velocity values (average ± S.E.M.) were not corrected for diffusion.

[b] Percent difference (average ± S. E. M.) between control velocity versus velocity in the presence of the compound tested for inhibition.

[c] Differences were determined to be not significant by the two-tailed t test.

Appendix 4 Steady state accumulation of SarCNU

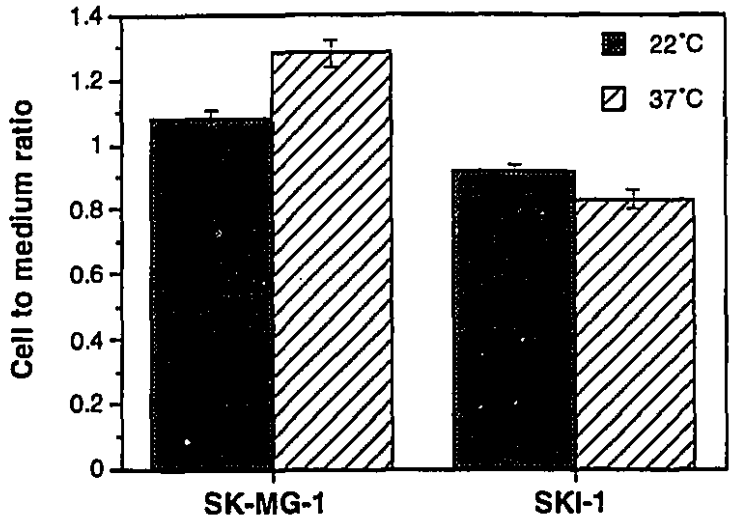


Figure A.4.1 Comparison of the steady state of accumulation of 50 µM [^{3}H]SarCNU by SK-MG-1 cells and SKI-1 cells at 22 and 37°C. The uptake, in suspension, is expressed as cell:medium distribution ratio as described in "Materials and Methods." Points, mean of 6 separate experiments performed in quadruplicate; bars, SE.

Appendix 5 Cytotoxicity of BCNU in SK-MG-1 and SKI-1 cells

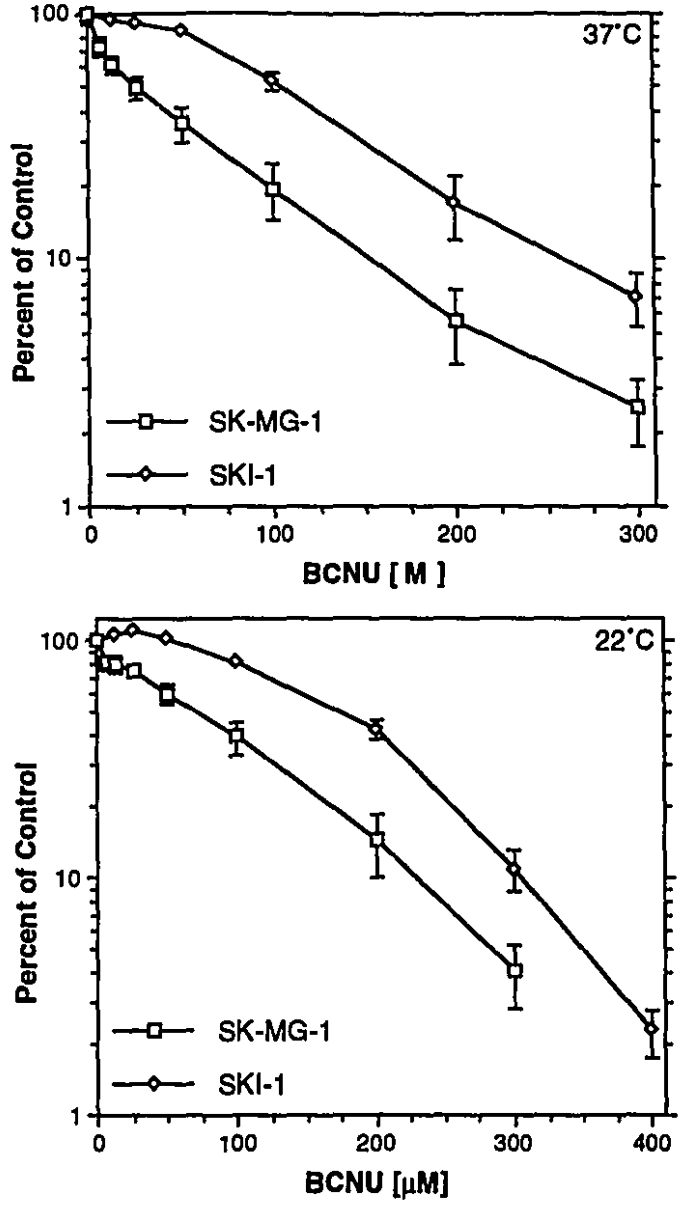


Figure A.5.1. Comparison of the cytotoxic activity of BCNU in SK-MG-1 cells and SKI-1 cells at 22 and 37°C in the SRB assay. The percentage of control SRB dye binding was plotted against the concentrations in μM of BCNU. Points, mean of 11 separate experiments for 37°C, and mean of 6 separate experiments for 22°C, with each drug concentration performed in quadruplicate; bars, SE.

Appendix 6 Calibration of cascade column chromatography

Materials and Methods

Calibration of Cascade Column Chromatography for Norepinephrine and Metabolites. The procedure for preparing Alumina (Brockman II-III) and Dowex 50Wx4 (200-300 mesh) has been previously described [519, 520]. Alumina (Brockman II-III) is now available, commercially, and does not have to be prepared. (-)-Norepinephrine, NMN, DOPEG, MOPEG, VMA and DOMA were dissolved, individually, in 0.01 N HCl to a final concentration of 100 µM and stored at -20°C until utilized. Each catechol was assayed separately by the addition of the catechol to 1 ml of 2 M Na acetate pH 8.2 to a final concentration of 50 µg/ml. The samples were then applied to Alumina columns equilibrated with 2 M Na acetate pH 8.2 and the procedure of cascade column chromatography was carried out as previously described [519]. Samples were collected and examined for native fluorescence (uncorrected 280/340 nm excitation) with the Sim·Aminco SPF-500C spectrofluorometer. The fractions should contain: Fraction I, VMA and MOPEG (O-methylated and deaminated); Fraction II, NMN; Fraction III, DOPEG; Fraction IV, norepinephrine; Fraction V, DOMA; and the 0.5 N acetic acid wash through which should contain primarily low amounts of norepinephrine, DOPEG and DOMA. Each compound was analyzed 3 times by this technique and results tabulated to ensure that the procedure was able to separate each catechol compound.

[7-^{3}H]-(-)-Norepinephrine (11.9 mCi/mmol) was separated by this technique to verify purity and was determined to be greater than 95% pure.

Results

Following separation of norepinephrine and its metabolites by cascade column chromatography, it was verified that each fraction contained significant amounts of the appropriate catechol (see Table 1). In addition, the analysis of [7-^{3}H]-(-)-norepinephrine by this technique verified that fraction IV contains norepinephrine with little contamination of other fractions.

Table A.6.1 Summary of fluorometry of catechol compounds separated by cascade chromatography

	Fraction[a]						
Catechol	I	II	III	IV	V	0.5 N Acetic Acid	%[b] Recovery
MOPEG	83 ± 9	0	0	2 ± 1	4 ± 2	6 ± 2	95 ± 11
VMA	95 ± 4	3 ± 2	4 ± 4	0	2 ± 1	5 ± 2	106 ± 11
NMN	0	89 ± 6	1 ± 1	0	1 ± 1	0	91 ± 7
DOPEG	1 ± 1	0	60 ± 5	0	13 ± 6	10 ± 2	85 ± 4
NE	4 ± 4	9 ± 9	0	69 ± 4	2 ± 1	2 ± 1	83 ± 6
DOMA	8 ± 1	0	9 ± 2	10 ± 1	72 ± 7	12 ± 6	110 ± 5

[a] Number of experiments is 3 for each compound.
[b] Percent of the starting material recovered.

Discussion

Each fraction was verified to contain primarily the catechol metabolite expected as discussed by the designers of the cascade

chromatography technique [519]. However, there was some cross contamination of other fractions. This contamination is not likely due to an inefficient technique since [7-^{3}H]-(-)-norepinephrine was found primarily in fraction IV. It is instead most likely due to the presence of impurities found in the nonradiolabeled compounds and difficulties encountered with background readings during spectrofluorometry.

Appendix 7 Initial velocity of (-)-norepinephrine influx into SKI-1 cells versus substrate concentration

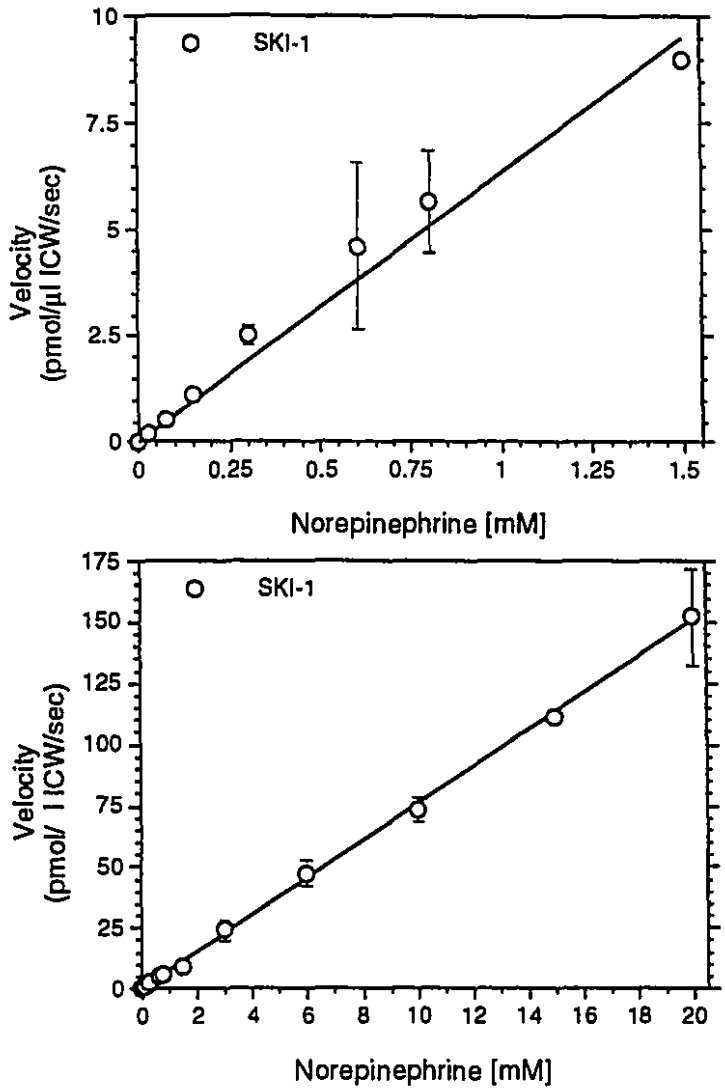


Figure A.7.1. Influx of [^{3}H](-)-norepinephrine into SKI-1 cells as a function of concentration in SKI-1 cells. The 5 second uptakes of SarCNU from (A) 25 µM to 1.5 mM and (B) 25 µM to 20 mM were measured in suspension at 37°C, as described in "Materials and Methods." Points, mean of at least 4 separate experiments with each drug concentration performed in quadruplicate; bars, SE.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The following facts in this thesis represent original contributions to scientific knowledge:

1. Characterization of the catecholamine extraneuronal uptake$_2$ transporter in SK-MG-1 cells is the first direct demonstration of this transport system in mammalian glioma cell lines or cells of glial origin.

2. The transport of SarCNU into the human glioma cell line SK-MG-1 occurs through the catecholamine extraneuronal uptake$_2$ transporter albeit with a reduced affinity compared to the natural substrates of norepinephrine and epinephrine. This is the first direct demonstration of a carrier-mediated mechanism of uptake of a chloroethylnitrosourea.

3. The SarCNU-uptake$_2$ transporter was demonstrated to be responsible for the increased influx rate, steady-state accumulation and cytotoxicity of SarCNU in sensitive SK-MG-1 cells versus resistant SKI-1 cells.

4. A reduction of temperature from 37 to 22°C was sufficient to reduce the efficiency of carrier-mediated transport of SarCNU into SK-MG-1 cells such that there was no demonstrable SarCNU-uptake$_2$ transporter.

5. Sarcosinamide is able to significantly reduce the area under the curve for SarCNU accumulation over one hour which correlates well with its ability to inhibit the initial rate of SarCNU influx and the *in vitro* cytotoxicity of SarCNU against SK-MG-1 cells.

6. The apparent lack of the SarCNU-uptake$_2$ transporter in the human glioma cell line SKI-1 was demonstrated to be responsible for the reduced steady state accumulation and reduced cytotoxicity of SarCNU in resistant SKI-1 cells versus sensitive SK-MG-1 cells.

7. The resistance of SKI-1 cells to SarCNU compared to SK-MG-1 is a combination of reduced transport by the SarCNU-uptake$_2$ transporter and innate resistance mechanisms as yet not characterized.

8. Calculations using experimentally obtained kinetic parameters revealed that at physiological concentrations of norepinephrine, the rate of carrier-mediated transport of SarCNU from 1 μM to its theoretical PPC will not be significantly affected and vice versa.

9. This investigation demonstrates that the presence of sarcosinamide, an amino acid amide, on the N-3 portion of a chloroethylnitrosourea presents a structure that causes SarCNU to be recognized and transported by the extraneuronal catecholamine uptake$_2$ transporter. This transport may therefore be responsible for the increased level of cytotoxicity of SarCNU against human gliomas *in vitro* and *in vivo*.

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