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The catecholamine extraneuronal uptake$_2$ transporter is associated with the increased sensitivity of gliomas to sarcosinamide chloroethylnitrosourea

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August, 1997

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master's of Science

Abstract

SarCNU, a novel chloroethylnitrosourea analogue, is transported by the extraneuronal uptake$_2$ transporter (uptake$_2$). SK-MG-1 human glioma cells are sensitive to SarCNU cytotoxicity and express uptake$_2$ whereas SKI-1 glioma cells have no detectable transporter, and are relatively resistant. To clone uptake$_2$, we detected differences in RNA expression utilizing differential display. With differential display, we detected a novel sequence expressed in SK-MG-1 cells but not in SKI-1 cells, having 62% homology to an expressed sequence tag clone from human brain, and could be a partial sequence of uptake$_2$. In the treatment of SF-295 glioma xenografts in athymic mice, SarCNU had superior activity than 1,3-bis-(2-chloroethyl)-1-nitrosourea. This suggested that SF-295 cells express uptake$_2$. We determined if expression of uptake$_2$ in the established SF-295 cell line correlated with the enhanced activity of SarCNU *in vivo*. Transport of [^{3}H]SarCNU was not decreased by inhibitors of uptake$_2$ in the SF-295 cell line and its steady state accumulation was similar to that of SKI-1. The increased stability of SarCNU versus BCNU may account for its enhanced activity *in vivo* or the expression of uptake$_2$ *in vivo* may differ from its expression in vitro.

Résumé

(2-Chloroéthyl)-3-sarcosinamide-1-nitrosourée (SarCNU) est un analogue expérimental de la chloroethylnitrosourée. Transfert$_2$, décrit initialement pour le transport des catécholamines, permet le transport de SarCNU. SK-MG-1, une lignée cellulaire humaine d'origine gliale, est sensible à SarCNU et exprime transfert$_2$. Cependant, transfert$_2$ semble être absent dans la lignée cellulaire d'origine gliale SKI-1 qui démontre de plus une résistance à SarCNU comparativement aux cellules SK-MG-1. Afin de cloner transfert$_2$, nous avons exploité les différences d'expression de l'ARN entre les lignées SK-MG-1 et SKI-1 en utilisant la technique de 'differential display'. Ceci nous a permis d'identifier une nouvelle séquence exprimée uniquement dans la lignée cellulaire SK-MG-1 et démontre 62% d'homologie à une séquence tirée d'une librairie de tissu cérébrale humain. SarCNU a une activité anti-tumeur supérieure à celle de la 1,3-bis-(2-chloroéthyl)-1-nitrosourée (BCNU) dans le traitement des xénographes gliomes SF-295 implantées dans des souris athymique. Ceci suggère que transfert$_2$ est exprimé dans les cellules SF-295. Nous avons déterminé s'il y avait une corrélation entre l'expression du transfert$_2$ dans la lignée cellulaire établie SF-295 et l'activité supérieure de SarCNU *in vivo*. Le transport de [^{3}H]SarCNU n'était pas diminué par des concentrations excessives d'inhibiteurs du transfert$_2$ dans la lignée cellulaire SF-295 et l'accumulation maximale de [^{3}H]SarCNU dans SF-295 était similaire à celle observée avec SKI-1. L'activité supérieure de SarCNU contre BCNU *in vivo* pourrait être d'une part le résultat de la stabilité supérieure de SarCNU comparativement à BCNU, ou d'autre part due à une différence entre l'expression de transfert$_2$ *in vivo* et de son expression *in vitro*.

Acknowledgments

I would like to express my appreciation to my supervisor, Dr. Lawrence Panasci, for his encouragement throughout my studies.

I am also very greatful to Camil Sayegh for translating my abstract and for the critical review of this work. I could not have done it without your enormous amount of support and patience.

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Chapter 3

Transport of [^{3}H]SarCNU and [^{3}H]NE in the SF-295 human glioma cell line

List of Abbreviations

3-MADG: 3-methyladenine-DNA-glycosylase

ACNU: 1-[(4-amino-2-methylpyrimidin-5-yl)methyl]-3-(2-chloroethyl)-3-nitrosourea hydrochloride

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

CCNU: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea

CENU: chloroethylnitrosourea

CHO: Chinese hamster ovary cells

CNS: central nervous system

CNU: N-(2-chloroethyl)-N-nitrosourea, or 1-(2-chloroethyl)-1-nitrosourea

COMT: catechol-O-methyltransferase

Decynium 22: 1,1'-diethyl-2,2' cyanine

Disprocynium 24: 1,1'-diisopropyl-2,4'-cyanine

ENU: 1-ethyl-1-nitrosourea

ERCC: excision-repair cross complementing

GBM: glioblastoma multiforme

GSH: glutathione, γ-glutamylcysteinylglycine

GST: glutathione-S-transferase

ISCL: interstrand cross-link

MAO: monoamine oxidase

Mer+: methylation repair positive

Mer-: methylation repair minus

MGMT: O^6-methyguanine-DNA methyltransferase

MPP+: 1-methyl-4-phenylpyridinium

NE: norepinephrine

NER: nucleotide excision repair

PAG: Dulbecco's incomplete phosphate-buffered saline supplemented with 0.7% BSA fraction V, 0.25% dextrose and 0.001% phenol red, pH 7.4

PPC: peak plasma concentration

Sarcosinamide: N-methyl glycinamide

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

$Uptake_1$: neuronal catecholamine transport

$Uptake_2$: extraneuronal catecholamine transport

Chapter 1

General introduction and review of literature

1.1 General introduction

Chloroethylnitrosoureas [(CENU); 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1-[(4-amino-2-methylpyrimidin-5-yl) methyl]-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU)] are among the most widely used anti-tumor agents in the treatment of malignant gliomas. BCNU is one of the most effective single agents (1). Utilization of CENUs is complicated by the fact that 40 to 60% of gliomas are innately resistant to these drugs and even those that initially respond can develop resistance during the course of therapy (2,3). Treatment with CENUs induces delayed and cumulative myelosuppression which is dose limiting (4). Furthermore, CENUs do not produce long-term responses and even in combination with radiotherapy, do not significantly improve the long term survival of most patients (5). Therefore, the development of novel agents with increased tumoricidal activity and decreased toxicity is necessary for the successful treatment of tumors of the central nervous system (CNS).

(2-Chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) is a novel CENU analogue containing N-methylglycinamide (sarcosinamide) attached at the N3 position of the compound (6). The presence of this side group alters the pathway of decomposition of SarCNU at physiologic pH, and is the basis of its unique physico-chemical and biological properties as compared to all clinically available CENUs. Previous studies

using SK-MG-1 and SKI-1 human glioma cell lines have indicated that SarCNU is a substrate for the extraneuronal catecholamine transporter (uptake$_2$) (7-9). SK-MG-1 and SKI-1 cell lines stem from a glioma specimen of an untreated patient however SK-MG-1 cells are sensitive to SarCNU cytotoxicity and express uptake$_2$ whereas SKI-1 cells, which have no detectable transporter are relatively resistant (8, 10). The physiological role of uptake$_2$ is to transport released catecholamines across the membranes of postsynaptic cells, ultimately leading to their metabolic inactivation (11). SarCNU exploits this physiological process of tumor cells, resulting in an increased intracellular accumulation of the drug (7-9). *In vivo* studies have demonstrated that SarCNU is a more efficacious chemotherapeutic agent than BCNU in the treatment of subcutaneously implanted SF-295 and U-251 human glioma xenografts in athymic mice. SarCNU retained excellent antitumor activity at 66% and at 45% of its optimal dose, suggesting potential efficacy in humans (12). SarCNU is to undergo phase I clinical trials in the near future.

1.2 **Brain tumors**

1.2.1 Definition

The term "glioma" refers to tumors of glial cell origin. These include astrocytomas, oligodendrogliomas, ependymomas and gliomas with mixed cell populations (13). Gliomas are the most common primary neoplasms in the CNS and annually account for about 2.5% of all cancer deaths (14). These tumors constitute the 2nd most common pediatric cancer and are the 3rd leading cause of cancer related deaths in patients between 15 and 34 years of age (15). Gliomas are one of the most lethal human cancers, with a survival rate at 2 years being less than 20% for malignant tumors (16).

1.2.2 Grading

Malignant gliomas are divided into three grades, low grade astrocytoma, anaplastic astrocytoma and glioblastoma multiforme (GBM), based on the presence or absence of hypercellularity, vascular hyperplasia, mitoses, nuclear atypia, and necrosis (14). A strong correlation exists between age and degree of histological malignancy; low grade astrocytomas occur mostly in children whereas malignant forms are frequent in the elderly (17). Age, tumor grade and tumor type closely correlate with prognosis (13).

Low grade astrocytomas arise in the cerebral hemispheres and are the slowest growing glioma (18). Astrocytes, the stellate supporting cells of the brain and spinal cord located in the white matter, are believed to be

the progenitors of astrocytomas. Low grade astrocytomas are characterized by the presence of increased cellularity, mild to moderate nuclear pleomorphism, enlarged nuclei and prominent chromatin granules (14). These tumors are diffusely invasive, with invasion limited to the white matter. They account for 10 - 15% of primary brain tumors in adults, and result in a median survival of approximately 7.5 years following diagnosis (17). Glial fibrillary acidic protein is a marker for tumors of astrocytic lineage. Similar to low grade astrocytomas, anaplastic astrocytomas are characterized by increased cellularity, nuclear and cellular pleomorphism and are diffusely infiltrative. Anaplasia is distinguished by elevated mitotic activity and the presence of proliferative vascular changes. Pathologically, the cytoplasm of these tumors may be meager with nuclear lobation and enlargement (14). GBM is the most frequent and malignant astrocytic glioma. These tumors can arise *de novo* or by the progression of lower grade gliomas (18). Morphologically, they are largely variable, with the presence of both low and high grade features (19). The presence of necrosis, resulting from an insufficient blood supply to the tumor, is essential for the diagnosis of GBM. Microvascular proliferation, high cellularity and mitotic activity, with a growth fraction between 5 - 25% is a histological hallmark of these tumors (18). GBM can widely infiltrate the brain and spread to distant locations connected by white matter tracts, giving the appearance of multifocal glioma (14). GBM produces a

marked increase in intracranial pressure and results in a survival at two years not exceeding 20% (16, 17).

1.2.3 Chemotherapy of malignant gliomas

Malignant gliomas are one of the most resistant tumors to all treatment modalities. The standard therapy for malignant gliomas involves surgery, radiotherapy and chemotherapy. Surgical intervention in patients with high grade gliomas results in a median survival of four to six months. Complete resection of the tumor is not possible without causing excessive neurological damage to the patient. This is a consequence of their infiltrative growth (20). Adjuvant radiotherapy increases the median survival of patients to about thirty six weeks whereas the combination treatment of chemotherapy, radiotherapy and surgery increases survival to fifty-one weeks (5). The CENUs BCNU, CCNU and ACNU are the most active agents in the treatment of malignant gliomas (1). These drugs are non-ionized and relatively lipid-soluble, which enables them to cross the blood brain barrier (21). BCNU is one of the most active single drugs used in the treatment of gliomas with a 20-30% response rate (22). The effective clinical use of CENUs in the treatment of gliomas is limited by the fact that greater than 60% of brain tumors are innately resistant to CENUs. Even those that initially respond can acquire resistance during the course of treatment (2,3). Utilization of the CENUs induces pulmonary toxicity and delayed and cumulative bone marrow toxicity,

resulting in the depletion of hematopoietic lineages (23). Despite these facts, a meta analysis of 19 randomized trials indicated that there was a small but statistically significant short term survival advantage for glioma patients treated with radiation in combination with adjuvant BCNU as compared to patients receiving radiotherapy alone. Patients with anaplastic astrocytomas benefited most from this therapy (24). Whether post irradiation BCNU chemotherapy benefits patients with GBM is uncertain, however many large cooperative trials use adjuvant BCNU as part of the standard treatment (25).

Chemotherapy has had a limited effect on survival in patients with high grade astrocytomas. Poor drug delivery to the tumor, low tumor growth fraction, cellular resistance and heterogeneity results in the failure of chemotherapy (26). The blood-brain barrier poses a specific challenge as it is impermeable to most antineoplastic agents, which limits their entry into the brain parenchyma, making it difficult to deliver drugs in tumoricidal concentrations (27). Several new chemotherapeutic protocols have been utilized experimentally in combination with optimal surgical and radiation therapy to improve the initial treatment of malignant gliomas (25). Adjuvant chemotherapy with novel antineoplastic agents has been tested. Among the new agents, temozolomide was a promising experimental drug with 40-50% of patient with newly diagnosed and recurrent high grade astrocytomas having partial responses (1). Thus far, this drug has not been shown

to be more effective than BCNU. Combination chemotherapy combines the use of drugs which have different mechanisms of action. Patients with anaplastic astrocytomas treated with procarbazine, CCNU and vincristine had a survival rate two times greater than those who received BCNU alone (151.1 weeks versus 82.1 weeks respectively) (28). Procarbazine, CCNU and vincristine did not significantly improve survival of patients with GBM when compared to those receiving post irradiation BCNU (28). Approaches to maximize delivery of drugs to brain tumors utilizing intra arterial administration and high dose adjuvant chemotherapy in conjunction with bone marrow or stem cell support has been explored (29,30). However, the role of these strategies has not been clearly defined at this time.

1.3 **Chloroethylnitrosoureas**

1.3.1 Chemical activity of chloroethylnitrosoureas

CENUs have a broad spectrum of anticancer activity. They are used to treat brain tumors, lymphomas and other systemic malignancies (31). The observation that N-methyl-N'-nitro-N-nitrosoguanidine demonstrated antitumor activity against the L1210 cells in a murine model led to the development of haloethylnitrosoureas (32). The parent compound of the CENUs is N-(2-chloroethyl)-N-nitrosourea (CNU). Addition of distinct side groups to the N3 position of the parent drug gives rise to chemically distinct compounds.

At physiological pH, the CENUs undergo base catalyzed hydrolysis (33) (figure 1.1). The section of the CENU attached at the N1 position yields a chloroethyl diazohydroxide intermediate. The diazohydroxide can either undergo low energy SN2 reactions or can undergo a spontaneous rearrangement which is coordinated with loss of a nitrogen atom to yield a 2-chloroethyl carbonium ion, a chloronium ion or the 1-chloroethylcarbonium ion. The 2-chloroethyl carbonium ion alkylates cellular DNA through an SN1 mechanism (34). The portion of the CENU attached at the N3 position forms an organic isocyanate which carbamoylates the α-amino groups of amino acids, the ε-amino group of lysine and terminal amino groups of proteins (35). All CENU analogues produce the chloroethyl diazohydroxide following chemical decomposition. However the organic isocyanate differs in function with the side group attached at the N3 position. The stability as well as the carbamoylating potential of CENUs is determined by the N3 side chain.

1.3.2 (2-Chloroethyl)-3-sarcosinamide-1-nitrosourea

SarCNU is a novel CENU analogue containing sarcosinamide attached at the N3 position of the compound (figure 1.1) (6). This compound was originally synthesized with the intention that the sarcosinamide side group would facilitate uptake by the amino acid transport system of tumor cells (6). Unlike most nitrosoureas, SarCNU has excellent water solubility

Figure 1.1

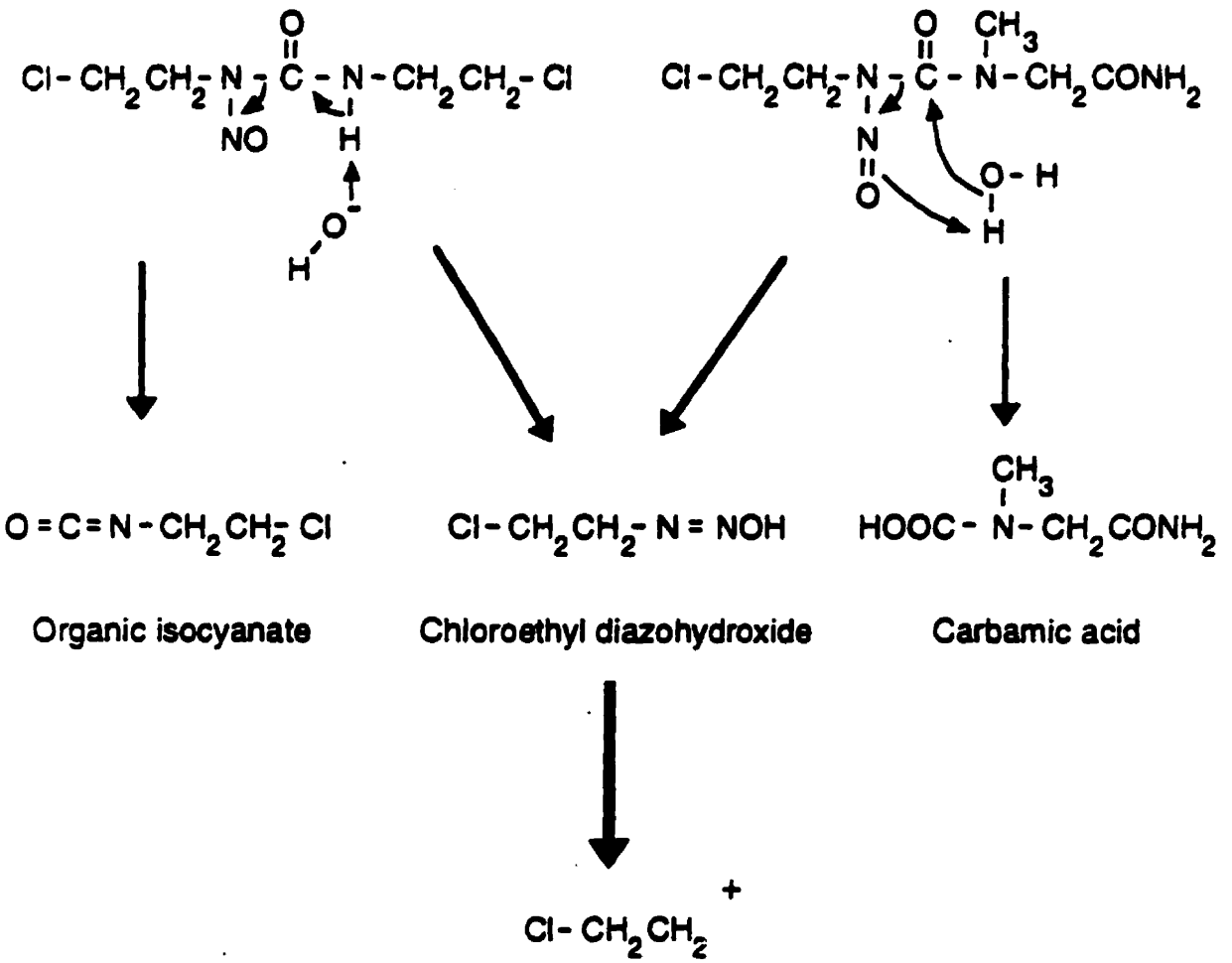
Chemical decomposition of CENUs. Following chemical breakdown, both BCNU and SarCNU produce a chloroethyl diazohydroxide ion from the N-1 atom. BCNU produces an organic isocyanate from the N3 atom, whereas SarCNU produces carbamic acid. The chloroethyl diazohydroxide can react by an SN2 mechanism or can undergo a spontaneous rearrangement to yield the chloroethyl carbonium, which aklylates cellular DNA by an SN1 mechanism. Figure modified from reference 39

BCNU

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

SarCNU

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



(>25mg/ml), which is due to its chemical structure and not to its ionization potential (36). To date, SarCNU is the most stable CENU analogue, having a chemical half life of about 333 minutes at pH=7.4, 37°C, which is six fold more stable than BCNU under similar conditions (6, 37). The decomposition of SarCNU is pH dependent. It undergoes acid and base catalysis below pH 3 and above pH 7 respectively and is most stable at acidic pH (36). The mechanism of decomposition of SarCNU is unique (Figure 1.1). The proposed mechanism of breakdown involves hydrolysis at the carbonyl carbon of the nitrosourea portion of SarCNU, generating a chloroethyl diazohydroxide ion from the N-1 atom and carbamic acid from the N3 atom. The carbamic acid further degrades into carbon dioxide and sarcosinamide. The production of carbamic acid results from the steric hindrance imparted by the methyl group at the N3 position, which alters the standard route of base catalyzed hydrolysis (6).

All clinically available CENUs enter tumor cells by passive diffusion (38). The presence of sarcosinamide alters the mechanism of SarCNU transport from passive diffusion to a combination of a saturable (facilitated) and non-saturable (non-facilitated) uptake (7). The saturable component of SarCNU uptake into tumor cells occurs by the extraneuronal uptake$_2$ transporter which is associated with an increased intracellular concentration of the drug, and may mediate the sensitivity of tumor cells (7-9, 39). *In vivo* experiments utilizing a subcutaneously implanted SF-295 CNS tumor

xenograft model in athymic mice demonstrated that SarCNU was more efficacious than BCNU in the treatment of these tumors using intravenous, intraperitoneal and oral routes of administration with daily or intermittent dosing schedules (12). In all cases, SarCNU cured more animals and was less toxic than BCNU at its optimal dose. In addition, SarCNU retained high antitumor activity at 66% and at 45% of its optimal dose, whereas BCNU showed a progressive loss of antitumor activity at lower doses. The possible implication of uptake$_2$ on the antitumor activity of SarCNU *in vivo* was demonstrated by the finding that intermittent dosing produced a high cure rate whereas a single bolus dose produced no tumor free animals. It was hypothesized that a single high dose saturates the uptake$_2$ transporter, leading to the inefficient accumulation of SarCNU into tumor cells, whereas multiple daily doses enable a more efficient accumulation of SarCNU (12).

1.3.3 Mechanism of chloroethylnitrosourea antitumor activity

Alkylation of DNA is the basis of the antitumor activity of CENUs. CENUs with low carbamoylation potential preserve their antitumor activity, whereas alkylation deficient nitrosoureas have reduced antitumor activity (40, 41). BCNU, CCNU and methylcyclohexyl chloroethylnitrosourea, all clinically utilized CENUs, produce similar types of DNA modifications. They form approximately 92% N^7G monoadducts (42), 3.5-4% N^7G-N^7G intrastrand crosslinks, and about an

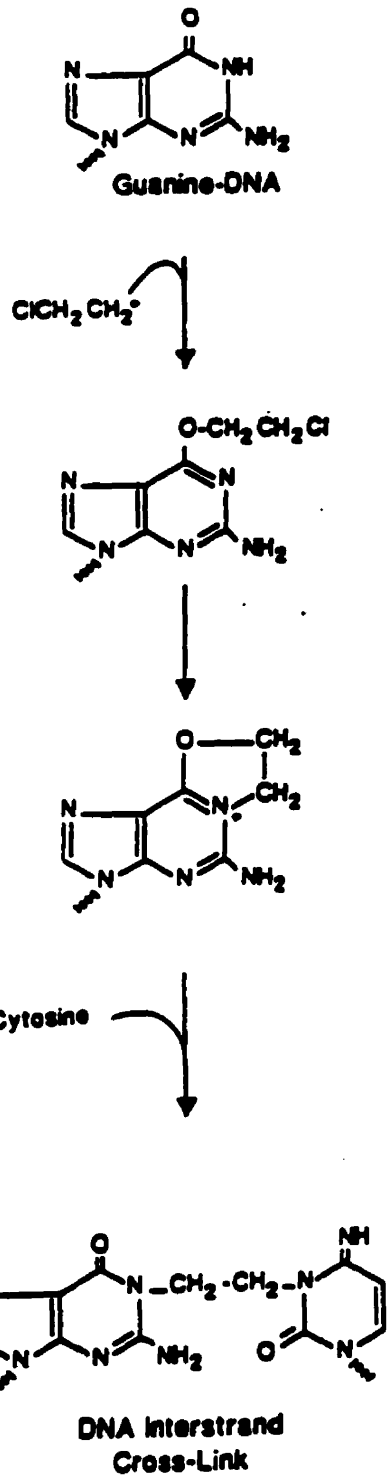
equal percentage of N^3C-N^1G interstrand crosslinks (ISCL) (43). Although ISCL account for a small percentage of DNA modifications, they are the most important mechanism by which CENUs mediate tumor cell kill (43, 44). ISCL ultimately induce cell death by interfering with cellular transcription and replication, by disrupting the double helical structure of DNA. This is supported by the finding that CENU treatment of cells leads to a prolonged S phase and a G2 arrest in the cell cycle (45). The relative cytotoxicity induced by the intrastrand crosslink is not clear. Carbamoylation does not significantly contribute to the anti-tumor effect of CENUs. However it has been shown to inhibit RNA processing, strand rejoining in DNA repair (46,47) and inhibits the ligase activity in excision repair (40, 48).

1.3.3.1 Mechanism of interstrand cross-link formation

ISCL are the product of a 2 step reaction (Figure 1.2). The first step involves an initial attack of the chloroethyl carbonium ion at the O^6 position of guanine on one DNA strand (49). The initial alkylation occurs rapidly, mostly within the first hours following exposure to the CENU, and leads to a chloroethylated product (49). Initial alkylation of O^6-guanine is concordant with the observation that cells proficient in the removal of O^6-methylguanine monoadducts, and by extension O^6-alkylguanine monoadducts, form fewer ISCLs

Figure 1.2

Mechanism of 1-[N^3-deoxycytidyl],2-[N^1-deoxyguanosyl]-ethane interstrand cross link. The first step in ISCL formation is the attack by the chloroethyl carbonium ion at the O^6 position of deoxyguanosine. The chloride ion is displaced by a nucleophilic site which leads to an intermolecular rearrangement, forming the N^1-O^6-ethanoguanine intermediate. The exocyclic bond of the N^1-O^6-ethanoguanine intermediate is cleaved, then reacts with the N^3 position of deoxycytidine to form 1-[N^3-deoxycytidyl],2-[N^1-deoxyguanosyl]-ethane ISCL. Figure modified from reference 51.


Guanine-DNA
ClCH2 CH2
O-CH2 CH2 Cl
Cytosine
DNA Interstrand
Cross-Link

than cells that are deficient in this process (50). The second step of ISCL formation proceeds in the absence of the free drug (49). There is a displacement of a chloride ion by a nucleophilic site on the opposite strand or the N-1 of the same guanine which is followed by an intermolecular rearrangement, forming an N^1-O^6-ethanoguanine intermediate (43). Cleavage of the exocyclic C-O bond occurs, followed by a reaction with N^3 of cytosine through an ethane bridge on the opposite DNA strand, resulting in a 1-(N^3-deoxycytidyl),2-(N^1-deoxyguanosyl)ethane ISCL (42,43). Incubation of O^6-methylguanine-DNA-methyltransferase (MGMT) with CENU-chloroethylated DNA resulted in a covalent association between the DNA and MGMT, with the structure of N1-(guan-1-yl)-2-(cystein-S-yl) ethane, supporting the existence of the N^1-O^6-ethanoguanine cross link intermediate (51). The cross linking reaction occurs over a period of hours, with maximum cross-linking detected between 6 and 12 hours (52).

1.3.4 Antitumor activity of SarCNU versus BCNU

In the human tumor cloning assay, SarCNU was more active than BCNU in the treatment of primary gliomas and glioma cell lines at equimolar concentrations. In a similar study, SarCNU at its calculated peak plasma concentration (PPC) of 68 μM reduced colony growth to ≤30% of control in ten of a total of thirteen glioma specimens and one of a total of three glioma cell lines, whereas BCNU at its clinically achievable PPC of 8 μM was active in one of a total of

thirteen glioma specimens and none of the glioma cell lines (10). Drugs reducing colony growth to ≤30% of control in the *in vitro* human tumor cloning assay have an excellent chance of inducing a response in patients from which the tumor was obtained (53). Using human bone marrow in the colony forming unit-C granulocyte-macrophage assay, SarCNU was seven fold less myelotoxic *in vitro* than BCNU (54). In the intracranial glioblastoma mouse model, BCNU produced 60% long term survivors at its maximal tolerated dose, whereas SarCNU resulted in 90 - 100 % long term survivors at its maximal tolerated dose and at 60% of that dose (55). In the mouse model, SarCNU was about ten fold less toxic systematically than BCNU, which correlated with the increased chemical stability of SarCNU (6, 55).

1.4 **Biological transport of SarCNU**

1.4.1 Transport of [^{3}H]SarCNU in SK-MG-1 and SKI-1 cells

All clinically available CENUs enter tumor cells by passive diffusion (38). Transport of SarCNU into SK-MG-1 occured by a mechanism other than passive diffusion and was supported by the finding that excess sarcosinamide in an *in vitro* cytotoxicity assay decreased the ED_{30} value of SarCNU, but not that of BCNU in SK-MG-1 (37). Transport of SarCNU was determined in SK-MG-1 cells. Influx of [^{3}H]SarCNU was temperature dependent, sodium independent and saturable. Initial rates of [^{3}H]SarCNU uptake were more rapid at 37°C than at 22°C and the saturable component of [^{3}H]SarCNU

transport followed Michaelis-Menten kinetics. Excess unlabeled SarCNU partially inhibited the initial rate of [^{3}H]SarCNU influx. Epinephrine inhibited the uptake of [^{3}H]SarCNU competitively, with a ki = 163 ± 15 μM. This is similar to the Km of epinephrine influx into SK-MG-1 cells. The initial rate of SarCNU accumulation into SK-MG-1 did not proceed against a concentration gradient nor was it inhibited by metabolic poisons, indicating that an energy source was not essential for the carrier mediated transport of SarCNU in this cell line (7). Uptake of SarCNU was partially mediated by facilitated diffusion, which accounted for 61% of total SarCNU accumulation in SK-MG-1 at its theoretical PPC (56). Furthermore, the saturable component of SarCNU and norepinephrine (NE) accumulation was inhibited by epinephrine, sarcosinamide, papaverine, disprocynium-24 and cimetidine, while the uptake$_1$ inhibitor desipramine, did not alter influx of NE or SarCNU (9). These findings are all consistent with accumulation of SarCNU by the uptake$_2$ transport system in SK-MG-1 cells.

The influx of [^{3}H]SarCNU in SKI-1 cells was compared to that of SK-MG-1 cells. The initial rate of uptake at 2 seconds was faster in SK-MG-1 cells than in SKI-1 cells at 37°C. At 22°C, the initial rates of uptake were similar. Transport of [^{3}H]SarCNU was saturable at 37°C over a 1000 fold range of concentrations in SK-MG-1 cells and SarCNU influx was inhibited by excess SarCNU, sarcosinamide or epinephrine. This is in contrast to SKI-1 cells, where there was no

evidence of saturable transport of SarCNU. Compared to SK-MG-1, the steady state accumulation of 50 μM [^{3}H]SarCNU was decreased by 35% in SKI-1 cells at 37°C whereas at 22°C the steady state accumulations were similar. This was not due to altered drug efflux or metabolism of SarCNU in either cell line (8). It was also demonstrated that NE transport was temperature dependent in SK-MG-1 cells but not in SKI-1 cells (9). These findings were consistent with an increased accumulation of SarCNU in SK-MG-1 cells that was associated with the presence of the uptake$_2$ carrier, which was not detected in SKI-1 cells.

1.4.2 Cytotoxicity studies of SarCNU versus BCNU

In vitro cytotoxicity studies demonstrated that SKI-1 cells were 3 fold more resistant to SarCNU when compared to SK-MG-1 cells at 37°C. At 22°C, SKI-1 cells were only 2 fold more resistant, yet accumulation of [^{3}H]SarCNU was similar in both cell lines. The 2 fold increase in resistance observed in SKI-1 cells as compared to SK-MG-1 cells to SarCNU was similar to the 2 fold resistance to BCNU at 22°C and at 37°C (8). Although there was a difference in sensitivity to SarCNU when compared to BCNU at 37°C, both SK-MG-1 and SKI-1 were methylation repair minus (Mer-), expressed similar amounts of N^3-methyladenine-DNA-glycosylase (3-MADG) and the mRNA levels of mdr 1, ERCC-1 and gluthione-S-transferase (GST)μ were similar. In addition, SK-MG-1 cells had an eight fold greater glutathione (GSH) content than SKI-1 cells (57). The

increased sensitivity of SarCNU in SK-MG-1 cells was hypothesized to be secondary to its increased accumulation by uptake$_2$. SarCNU is the first CENU that has been demonstrated to enter tumor cells by carrier mediated uptake.

1.5 Proposed mechanisms of chloroethylnitrosoureа drug resistance

1.5.1 O^6-methylguanine-DNA methyltransferase

MGMT, a DNA repair protein, is the most thoroughly understood mechanism of CENU drug resistance. MGMT is constitutively expressed in normal cells and tissues (58). It constitutes a primary defense mechanism against the cytotoxic effects of CENUs and other alkylators (59). The gene encoding MGMT is situated on chromosome 10, which often displays a loss of heterozygosity in malignant gliomas (60). Its promotor contains CpG islands which are susceptible to methylation (61). The molecular content of MGMT largely varies among cell types. Liver and spleen are MGMT rich while brain tissue expresses low levels of the protein. Cells with detectable MGMT activity have a methylation repair positive (Mer+) phenotype whereas deficient cells are Mer-. The Mer- phenotype results from the lack of MGMT gene transcription, and is not due to a mutant or unstable protein (62).

The biological function of MGMT is to catalyze the irreversible transfer of small alkyl adducts from the O^6 atom of guanine to a cysteine residue within its structure. This

yields S-alkylcysteine within the protein and returns guanine to its unmodified form (51). The active site of MGMT is not regenerated therefore the number of repaired O^6 adducts is restricted to the MGMT content of the cell, and to the rate of resynthesis of the protein. The preferred substrate is O^6-methylguanine, however longer alkyl groups are repaired, but with lower efficiency (63). MGMT eliminates 2-chloroethyl groups from O^6-guanine and reacts with N^1,O^6-ethanoguanine, which is the precursor for CENU induced DNA-ISCL formation (51).

The cytotoxicity of CENUs correlates with DNA cross linking (64). Many Mer- tumor cell lines were found to have a greater number of ISCLs following CNU treatment than Mer+ cells (44, 65). Transfection of human MGMT into Mer- human and rodent cell lines rendered them Mer+, and increased cellular resistance by reducing the toxicity, mutagenicity and the number of sister chromatid exchanges induced by nitrosoureas (66-68). Expression of MGMT in clinical glioma samples and human glioma tumor cell lines correlated with decreased ISCL formation and resistance following BCNU treatment. Glioma bearing patients with high levels of MGMT, treated with BCNU, had a higher death rate than those with low MGMT, following similar treatments (69). Approximately 20% of human brain tumor lines are Mer- (62). Studies have suggested that the level of MGMT expression correlated with the graded methylation of both the promoter and the body of the MGMT gene (61), ie: increased methylation levels were associated

with decreased levels of MGMT (70). Quantitation of MGMT in normal brain tissue adjacent to a primary brain tumor indicated that 55% of the samples had no detectable MGMT activity. This event was thought to be mediated by epigenetic silencing (ie, by altered CpG methylation in the promotor and/or body of the gene) and may play a primal role in the development of some brain tumors (59). Wild type p53 has been shown to reduce cellular levels of MGMT by suppressing transcription of the gene (71).

Resistance to CENUs can be partially overcome by depleting cellular MGMT (72, 73). The free base inhibitor, O^6-benzylguanine, inactivates MGMT activity and enhances the sensitivity of tumor cells to alkylating agents (73). Clinical use of O^6-benzylguanine may be limited by its nonspecific inactivation of MGMT in normal tissue. However it is presently in phase I clinical trials and its potential to increase the therapeutic index of BCNU is currently being investigated. Other O^6-benzylguanine analogs such as O^6-benzyl-2'-deoxyguanosine, are presently being investigated (74).

1.5.2 Glutathione/Glutathione-S-transferase

Although the efforts of much research has been focused on glutathione/glutathione-S-transferase (GSH/GST), their role in CENU drug resistance is not completely understood. GSH is a ubiquitously expressed intracellular thiol tripeptide (75). Levels of GSH in human plasma range from 10

to 30 μM whereas tumors can contain between 1 and 10 mM of the protein (76). The dissociation of GSH at physiological pH yields a thiolate nucleophile that can spontaneously react with electrophilic sites or through enzyme mediated conjugation by GSTs. In mammalian cells, the GST supergene family of cytosolic isoenzymes consists of alpha, pi, mu, theta and sigma, based on isoelectric points (75). GSTs are part of the phase I (oxidation)/phase II (conjugation) system that metabolizes lipophilic compounds. The end point of this system is the conversion of lipophilic agents to more polar derivatives thereby facilitating their inactivation and elimination (77).

There has been substantial correlative data linking GSH and GST to tumor cell drug resistance. However, evidence indicating a direct metabolic role in CENU drug resistance is still lacking. Previously, it was demonstrated that GSH quenches chloroethylated DNA monoadducts, leading to the inhibition of cross link formation (78). This implies that GSH reacts with the cross link progenitor of chloroethylated DNA. BCNU was shown to be detoxified by a denitrosation reaction catalyzed by GSTs (79). Class μ isoenzymes were the best catalysts of *in vitro* denitrosation reactions in rat brain tumor cells (80). In the UWR-2 brain tumor cell line, a 4 times molar excess of GST preincubated with BCNU significantly inactivated the drug, resulting in a 32% decrease in cross link formation (81). In three human brain

tumor cell lines, resistance to alkylating agents was associated with variations in the GSH/GST system. In these cell lines, increased expression of GSH correlated with resistance to BCNU. It was also suggested that elevated GSTπ activity correlated with BCNU resistance in human malignant astrocytoma cell lines (82).

Butathionine sulfoximine, an irreversible inhibitor of γ-glutamyl cysteine synthetase, has been utilized to decrease GSH content in tumor cells. In some human brain tumor cell lines, butathionine sulfoximine treatment increased the sensitivity of these cells to BCNU (83). In contrast, there was little effect on BCNU induced sister chromatid exchanges and cytotoxicity in rat brain tumor cells pretreated with butathionine sulfoximine. A similar treatment increased cytotoxicity and the number of sister chromatid exchanges induced by nitrogen mustards (84). This suggests that GSH and GSTs may be important in the resistance of certain tumors in relation to alkylating agents, and yet do not seem to play a role in other tumors.

1.5.3 3-Methyladenine-DNA glycosylase

3-methyladenine-DNA glycosylase (3-MADG) is an enzyme involved in the base excision repair pathway. 3-MADG is most effective at removing N^3-methyladenine adducts although it removes a wide variety of alkylated bases including N^3-

methylguanine, N^7-methyladenine, as well as ethyl lesions (85-87).

A role for 3-MADG in repair and resistance to CENUs has been demonstrated by transfection experiments of *E.coli* glycosylase I (tag) and *S. cerevisiae* glycosylase (MAG) genes (88, 89). Transfection of Chinese hamster ovary cells (CHO), which are deficient in 3-MADG activity, with the *E.coli* tag gene increased survival of these cells by 55% following 1-methyl-1-nitrosourea treatment (88). Since activity of tag is restricted to the removal of 3-methyladenine, this suggests that the increased survival was secondary to the removal of this adduct. Furthermore, purified *E.coli* 3-methyladenine-DNA glycosylase II (AlkA) releases 7-(2-hydroxyethyl)guanine, 1,2-bis(7-guanyl) ethane, and 7-(2-chloroethyl)guanine from CCNU treated DNA (90). Its role in the removal of the highly cytotoxic N^3C-N^1G ISCL is undetermined. Additional evidence suggesting a role for glycosylase in CENU drug resistance was observed in *S. cerevisiae*. Disruption of the MAG gene in these cells increased the sensitivity to CNU whereas transfection of MAG into glycosylase deficient *E.coli* protected against CNU toxicity by releasing 7-hydroxyethyl guanine and 7-chloroethylguanine (89). A role for glycosylase has also been suggested in brain tumor cells. Extracts from the CNU resistant SF-188 glioma cells had a 2 to 3 fold higher glycosylase activity, determined by the release of methylated bases from DNA modified with [^{3}H]dimethyl sulphate, as compared to the sensitive glioma cell line SF-126 (91).

Transfection of human 3-MADG cDNA into wild type CHO AA8 cells and NER deficient CHO UV20 cells increased glycosylase levels. In contrast to *E.coli*, overexpression of 3-MADG in CHO cells did not increase resistance to BCNU or melphalan in the presence or absence of nucleotide excision repair (NER), suggesting that this enzyme has no role in CENU drug resistance (92). At this time, the role of 3-methyladenine-DNA glycosylase in relation to CENU tumor cell drug resistance remains unclear.

1.5.4 Nucleotide Excision Repair

NER is a multi-step reaction that eliminates a wide variety of structurally unrelated lesions including UV induced photoproducts and bulky DNA adducts, such as O^6-methyl guanine (93). Genetic and biochemical data in bacterial systems first suggested a role for NER in CENU drug resistance. In *E.coli*, loss of either uvr excision or recA dependent repair increased the sensitivity to CNU and BCNU versus wild type strains (94). Further evidence supporting a role for NER in CENU resistance emerged from studies in MGMT-negative CHO wild type and CHO UV41 cells, having defective NER activity due to a mutant ERCC-4 gene. Despite a lack of endogenous MGMT activity the UV41 cells were 20 fold more sensitive to CNU than their wild type counterpart. Transfection of MGMT resulted in about the same fold increase in resistance to CENU, yet the original difference in

sensitivity to CENU between the cell lines due to the excision repair defect persisted (67).

All rodent ERCC and XP mutant cell lines sensitive to UV radiation are also sensitivity to cross-linking agents (95). Rodent ERCC-4 and ERCC-1 are especially sensitive to mitomycin-C (96) whereas XPA mutant cell lines are highly sensitive to ENU (1-ethyl-1-nitrosourea) (97). This suggested that NER was involved in the repair of ethylated bases. It was shown that removal of ENU induced damage in NER proficient CHO cells occurred 30% faster in the transcribed strand as compared to the non-transcribed strand following exposure to ENU. In contrast, in CHO UV5 cells deficient in NER due to a mutant ERCC-2 gene, removal of N-ethylpurines was observed yet both strands were repaired at the same rate, most likely by base excision repair (97). This suggested that transcription-coupled repair of ethyl lesions required wild type ERCC-2. More recently, a correlation between ERCC-2 protein levels and the IC_{90} values of BCNU and SarCNU cytotoxicity and the IC_{50} values of UV light was demonstrated in human tumor cell lines (98). Since UV light induced damaged is classically repaired by the NER pathway, and that resistance to BCNU and SarCNU correlates with the resistance of UV light, this suggests that NER via ERCC-2 confers resistance to CENUs. At present, it has not been directly demonstrated that increased NER activity is involved in resistance to CENUs.

1.6 Extraneuronal catecholamine uptake$_2$ transporter

The catecholamines, epinephrine, NE and dopamine function in synaptic signal transmission in the CNS. An essential step in signal transmission is the irreversible metabolic inactivation of neurotransmitters following their release by reuptake into specialized transport systems located on the membranes of releasing neurons and adjacent non-neuronal cells (99-101). Two clearly distinct transport systems, neuronal catecholamine (uptake$_1$) and uptake$_2$, function to remove released catecholamines. Uptake$_1$ transports catecholamines from the extracellular space across the axonal membrane of adrenergic nerves whereas uptake$_2$ transports NE and related amines across the membranes of various postsynaptic cells (11). Uptake$_2$ is the first step in a metabolic pathway that is associated with O-methylation by catechol-O-methyltransferase (COMT) and to a lesser magnitude, deamination by monoamine oxydase (MAO) (101).

Uptake$_2$ was first identified in the isolated perfused rat heart by Iverson. The transporter was characterized as having a 1000 fold reduced affinity for NE compared to neuronal uptake$_1$ (99). Uptake$_2$ functions at all NE concentrations. However at low amine concentrations (up to 1 µmol) it is not readily detectable due to the rapid metabolism of accumulated catecholamines by COMT and MAO (102). At low concentrations of NE, COMT and MAO have not reached saturation and the system is an "irreversible, metabolizing site of loss". At high concentrations, the enzymes become

saturated and the system functions as a "reversible accumulating site of loss" (101).

The physiological role of uptake$_2$ is unclear. It is believed to function as "a second line of defense" protecting against overspilled neurotransmitter that have escaped uptake$_1$ (101). In certain pathological conditions involving elevated neurotransmitter release, such as epilepsy, uptake$_1$ can become saturated (11, 103). In these circumstances, uptake$_2$ may become the major mechanism of catecholamine uptake (103). The low rate of transport by uptake$_2$ *in vivo* is counteracted by the abundant number of cells which express the uptake$_2$ (104).

Uptake$_2$ is located primarily in sympathetically innervated tissues and organs which include myocardial cells, vascular smooth muscle (arteries, veins, and venules), and non vascular smooth muscle (trachealis, iris, spleen, intestine, and uterus) (99, 101, 105). The transporter was recently demonstrated in the human renal carcinoma cell line, Caki-1 and in FL human amnion cells (106, 107). The proximity of neurons and glia, which express significant amounts of COMT and MAO, in the CNS suggested that glial cells may play a role in the transport of released transmitters at the neuronal junction. Recently, uptake$_2$ was demonstrated in primary astrocyte cultures, several human glioma cell lines, and rat cerebral cortex slices suggesting that uptake$_2$ may inactivate catecholamines in the CNS in addition to peripheral organs (9, 108).

Uptake$_2$ is a saturable transport system (109). The biological substrates of uptake$_2$ in increasing order of affinity include dopamine < NE < epinephrine < isoprenaline (99). It has been demonstrated that uptake$_2$ is sensitive to O-methylated catecholamines, various corticosteroids, β-haloalkylamines, and N-substituted catecholamines (102, 110, 111). Guinea pig trachealis smooth muscle, rat heart and rat aorta demonstrate a small degree of substrate sterioselectivity, with preference for the (-)-isomer declining in the order isoprenaline > epinephrine > NE (112). There was no sterioselectivity in the rat heart and rabbit aorta for NE (101). Uptake$_2$ was not affected by changes in extracellular sodium, chloride or calcium. However it was affected by changes in K+, indicating that membrane potential was the driving force of uptake$_2$ (113, 114). Depolarization of the cell membrane leads to a decreased accumulation of NE, and hyperpolarization increases the accumulation. These observations led to the hypothesis that uptake$_2$ transports protonated species of its substrates, and is supported by the finding that the very potent inhibitors of uptake$_2$, 1-methyl-1-nitrosourea (MPP+) and 1,1'-diethyl-2,2'cyanine (decynium-22), are permanent positively charged compounds (115-118).

1.8 **Research Objectives**

The aim of this thesis project was to expand on two specific observations that have been previously made.

1- In view of the finding that SarCNU is a more effective chemotherapeutic agent compared to BCNU in the treatment of the SF-295 human glioma xenograft in athymic mice, we hypothesize that the SF-295 tumor expresses the extraneuronal $uptake_2$ transporter.

Therefore, we wanted to determine whether the $uptake_2$ transporter was expressed in the established SF-295 human glioma cell line *in vitro*, and whether this would correlate with the increased antitumor activity of SarCNU *in vivo*.

2- In view of the finding that SarCNU enters SKI-1 cells by passive diffusion, and that SarCNU transport is mediated partially by facilitated diffusion in SK-MG-1 cells, we hypothesize that SK-MG-1 expresses $uptake_2$ and that expression of the transporter is greatly decreased or absent in SKI-1 cells.

To initiate steps which could ultimately lead to cloning the $uptake_2$ transporter, we wanted to detect novel differences expressed in the SK-MG-1 cell line, yet diminished in expression in SKI-1 cells, utilizing the technique of differential display.

Chapter 2

Materials and methods

2.1 **Preface**

This chapter describes the materials and methods used in this thesis. The candidate was responsible for performing all the described techniques.

2.2 **Transport Studies (Chapter 3)**

2.2.1 Materials

RPMI 1640, fetal calf serum and Dulbecco's phosphate buffered saline were supplied by Canadian Life Technologies. (2-Chloroethyl)-3-[^{3}H]sarcosinamide-1-nitrosourea ([^{3}H]SarCNU, 342 mCi/mmol) was prepared by Amersham Laboratories, Buckinghamshire, England, using a technique described by Suami et al (6). [7-^{3}H](-)-Norepinephrine ([^{3}H]NE, 11.9 Ci/mmol) was purchased from Dupont NEN. [Carboxyl-^{14}C]inulin (3.2 mCi/mmol), tritiated water (1 Ci/ml), bovine serum albumin (Fraction V, powder) and Ecolite (+) scintillation fluid were purchased from ICN. Dextrose and NaCl were purchased from Fisher. (±)-Epinephrine HCl, sarcosinamide HCl, desipramine HCl and Tris base were provided by Sigma Chemical Co. Chlorinated phenylmethylpolysiloxane oil was purchased from PCR Inc, Gainesville, Florida, USA. SarCNU (NSC 364432) was obtained from Dr. T. Suami, Keio University, Japan and disprocynium-24 was a gift from Dr. Edgar Schomig, University of Würzburg, Germany.

2.2.2 Cell culture

SF-295 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 µg/ml gentamycin (Schering, Pointe Claire, Quebec, Canada), at 37°C in a humidified 5% CO_2 atmosphere. Confluent monolayers of cells were washed once with Dulbecco's incomplete phosphate-buffered saline,

supplemented with 0.7% BSA fraction V, 0.25% dextrose and 0.001% phenol red, pH 7.4 (PAG) and harvested with a rubber policeman. Cells were collected by centrifugation for 5 minutes at 1300 rpm, washed twice with PAG and resuspended at a working density of 4 X 10^6 cells/ml in PAG.

2.2.3 Transport of [^{3}H]SarCNU and [^{3}H]NE

A modified version of the "oil-stop" methodology was utilized to asses the transport of 50 µM [^{3}H]SarCNU (specific activity of 6.84 µCi/µmol) or 25 µM [^{3}H]NE (specific activity of 23.8 µCi/µmol) in the SF-295 cell line in suspension at 37°C, as previously described (8,9,119). Essentially, the cell suspension (4x10^6 cells/ml) and PAG were incubated for 15 minutes at 37°C prior to the experiment. An aliquot of [^{3}H]SarCNU or [^{3}H]NE was added to the PAG, hereafter called "labeled PAG", to a final concentration of 100 µM and 50 µM respectively. A 200 µl aliquot of labeled PAG was added to the surface of 1 ml of chlorinated phenylmethylpolysiloxane oil, in a 1.5 ml eppendorf tube. Transport was initiated by the rapid addition of 200 µl of cell suspension to the labeled PAG. At predetermined time points (0, 2, 3, 4, 6, 8, 10, 15, 20, 30, 40, and 60 seconds for [^{3}H]SarCNU transport and 0, 5, 10 and 15 seconds for [^{3}H]NE transport), the cells were separated from the media by centrifugation in a Brinkman-Eppendorf microcentrifuge for 30 seconds at 14,000 x g. Steady state accumulation at 30 minutes was also

determined. A sample of the media was collected for liquid scintillation counting. The remaining media and chlorinated phenylmethylpolysiloxane oil was aspirated and the tubes inverted. Contaminating media was eliminated by wiping the inner walls of the tubes twice with cotton tip applicators. The cell pellets were lysed in 100 µl of 4.0 M sodium acetate pH 4.0 for 30 minutes. The cell pellet was resuspended and placed into 10 ml Ecolite (+) scintillation fluid for scintillation counting.

The intracellular water space was determined as previously described (7-9). Essentially, tritiated water and [carboxyl-^{14}C] inulin was added to the cellular suspension in PAG. The mixture was centrifuged through chlorinated phenylmethylpolysiloxane oil, as described previously. [Carboxyl-^{14}C] inulin contamination, which represents the extracellular water space within a cell pellet, contributed to 0.3 ± 0.02 µl per 1 µl intracellular water space. From every time point, this media contamination of cell pellets was subtracted. Accumulation was expressed as a cell to medium ratio which represents the distribution of [^{3}H]SarCNU or [^{3}H]NE in 1 µl of the intracellular water space and 1 µl of the extracellular medium. Each time point was performed in quadruplicate and the average was calculated.

2.2.4 Inhibition of [^{3}H]SarCNU and [^{3}H]NE transport

Inhibition analysis was performed as described above except that osmotically adjusted inhibitor agents (10 mM SarCNU, 10 mM sarcosinamide, 10 mM epinephrine, 1 μM disprocynium-24 or 10 μM desipramine) were added to the labeled PAG. Inhibition was assessed at three seconds.

2.3 SRB cytotoxicity studies (Chapter 3)

2.3.1 Drugs

SarCNU (NSC 364432) was obtained from Dr. T. Suami, Keio University, Japan. BCNU was supplied by Bristol-Myers. The drugs were dissolved in 0.001 M citrate buffer, pH 4.0 and stored at -20°C until needed.

2.3.2 Cell culture

SF-295 and UW-28 human glioma cell lines were obtained from the National Cancer Institute. The cells were grown in RPMI 1540 medium supplemented with 10% fetal calf serum and 10 μg/ml gentamycin at 37°C in a humidified 5% CO_2 atmosphere.

2.3.3 Measurement of cytotoxicity

The cytotoxicity assay was a modification of the sulforhodamine B colorimetric anticancer-drug screening assay (120, 121). Essentially, 0.5 mls of SF-295 (4,500 cells/ml) or UW-28 cells (15,000 cells/ml) were seeded on flat bottom 24-well plates (Canadian Life Technologies) in media buffered with hepes. Following a sixteen hour incubation period, cells

were dosed with various concentrations of SarCNU or BCNU in 0.001 M citrate buffer. Controls were dosed with diluent. On day four, 1.5 mls of RPMI 1640 medium was added to each well. On day seven, the medium was removed and the cultures were fixed to the plastic substratum by the addition of 1.0 ml of a solution containing 10% trichloroacetic acid and 0.9% NaCl. This was followed by a one hour incubation at 4°C. The cells were washed with five exchanges of water to remove residual trichloroacetic acid, the plates were air dried, then stained for 30 minutes with 0.4% sulforhodamine B dissolved in 1% acetic acid. The sulforhodamine B was aspirated and unbound dye was removed by washing five times with 1% acetic acid. Bound sulforhodamine B was solubilized in 2 mls of 10 mM Tris base and the OD at 500 - 550 nm was determined. The wavelength was chosen such that maximum sensitivity was obtained, as previously described (120). CA-Cricket Graph III version 1.01 (Computer Associates International Inc., Islandia, New York) was utilized to determine the IC_{90} values by exponential curve fit of the linear portion of the curve.

2.4 **Differential Display (Chapter 4)**

2.4.1 Cell Culture

SK-MG-1 cells were a gift from Dr. G. Cairncross (University of Western Ontario, London, Ontario). SKI-1 cells were provided by Dr. J. Shapiro (Barrow's Neurological Institute, Phoenix, Arizona). FL and Caki-1 were purchased from the American Type Culture Collection. SKI-1, SK-MG-1 and Caki-1 were cultured in McCoy's 5A media whereas FL cells required minimal essential media. Culture media was supplemented with 10% fetal calf serum and 10 µg/ml gentamycin. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.4.2 RNA extraction

RNAzol B (Tel-Test Inc.) was utilized to extract total RNA from SKI-1, SK-MG-1, FL and Caki-1 cells. Cells were grown to 70% confluence on 100 mm^2 petri dishes (Canadian Life Technologies), washed with PBS, harvested with a rubber policeman, then collected by centrifugation for five minutes at 1300 rpm. An aliquot of 0.2 ml RNAzol B was added per 10^6 cells, and the RNA was solubilized by pipetting. Next, an aliquot of 0.2 ml chloroform per 2 mls of RNAzol B was added to the mixture, and the solution was vigorously shaken for fifteen seconds. The samples were incubated on ice for five minutes, then centrifuged for 15 minutes at 4°C, 12 000 x g. The aqueous layer containing the RNA was added to an equal volume of isopropanol. The RNA was precipitated for fifteen

minutes at -70°C, and recovered by centrifugation at 4°C, 12 000 x g for fifteen minutes. The RNA pellet was washed in 75% ethanol, then resuspended in DEPC treated water. Chromosomal DNA contamination was removed by DNase treatment. The samples were frozen at -70°C until needed.

2.4.3 Reverse transcription of RNA

Reverse transcription was carried out as previously described (122, 123). An aliquot of 0.2 µg freshly diluted total RNA (0.1 µg/µl) was reverse transcribed in the presence of 20 µM dNTP, 1X RT buffer and 0.2 µM of one of the following primers $T_{12}MA$, $T_{12}MT$, $T_{12}MG$ or $T_{12}MC$ (GenHunter Corporation, Brookline, MA). The reactions were introduced into a thermocycler (MJ Research Inc., Model PTC-100) and subjected to the following cycling parameters: 65°C for 5 minutes, 37°C for 60 minutes followed by 95°C for 5 minutes. An aliquot containing 100 units of MMLV reverse transcriptase was added to each tube after 10 minutes at 37°C, and gently mixed. The samples were stored at -20 until needed.

2.4.4 PCR amplification

PCR reactions were carried out as previously described (122, 123). Aliquots of 2 µl of cDNA were amplified in the presence of 2 µM dNTP, 0.2 µM of the appropriate $T_{12}MN$ ($T_{12}MA$, $T_{12}MT$, $T_{12}MG$ or $T_{12}MC$), 0.2 µM AP primer (GenHunter Corporation, Brookline, MA), 1X PCR buffer, 1 µl ^{35}S-dATP (1200 Ci/mmol,

New England Nuclear) and 1 unit of AmpliTaq (Perkin-Elmer Corp.), in a final volume of 20 µl. Core mixes containing dH_2O, PCR buffer, dNTP, ^{35}S-dATP and AmpliTaq were prepared to minimize pipetting errors. The samples were introduced into a thermocycler using the following cycling parameters: 94°C for 30 seconds, 40°C for 2 minutes, 72°C for 30 seconds, for 40 cycles, followed by 72°C for 5 minutes. The samples were stored at -20°C until needed.

2.4.5 Electrophoresis of differential display

Four microliters of DNA loading dye (95% formamide, 10 mM EDTA pH 8.0, 0.09% xylene cyanol FF and 0.09% bromophenol blue) were added to 7 µl of cDNA sample. The cDNA was denatured at 80°C for 2 minutes then loaded onto a 6% DNA sequencing gel. Electrophoresis was performed in 1X TBE buffer (50 mM Tris base, 50 mM boric acid, 10 mM EDTA.$2H_2O$) at 60 watts constant power until the xylene dye migrated to the bottom edge of the gel. The gel was blotted onto a Whatman 3 M paper, and was dried for 1.5 hours at 80°C. This was followed by autoradiography for 24 to 72 hours.

2.4.6 Reamplification of the cDNA probes

Reamplification of the cDNA probes was carried out as previously described (122, 123). The autoradiogram was aligned with the dried gel. cDNA bands overexpressed in SK-MG-1 samples were carved from the gel using a clean blade. Gel slices were soaked in 100 µl of dH_2O for 10 minutes in a 1.5

mls eppendorf tube. The cDNA was extracted from the gel slice by boiling for 15 minutes, then was centrifuged for 2 minutes at 12 000 rpm in a Brinkman-Eppendorf microcentrifuge. The supernatant was collected, and the cDNA was recovered by ethanol precipitation by the addition 10 µl of 3M sodium acetate, 5 µl of 10 mg/ml glycogen and 450 µl of 100% EtOH. The cDNA was precipitated at -80°C for 30 minutes, then centrifuged at 4°C, 14,000 x g for 10 minutes. The DNA pellet was washed with ice cold 85% EtOH, air dried and resuspended in 10 µl dH_2O. The primer combinations and PCR conditions utilized for reamplification of the cDNA probe were similar to those described above. However, 4 µl of the cDNA probe was reamplified in a total volume of 40 µl, using a concentration of 20 µM dNTP instead of 2 µM, and the ^{35}S-dATP was omitted. Thirty microliters of the samples were electrophoresed on a 1.5% low melt agarose gel to confirm reamplification. The remaining samples were stored at -20 for subcloning.

2.4.7 Northern analysis

2.4.7.1 Blotting

RNA was prepared for electrophoresis by adding a volume of 25 µl total RNA (60 µg) to 77.5 µl premix solution (200 µl 10X MOPS (0.2 M MOPS, 0.15 M sodium acetate, 0.01 M EDTA) 350 µl 37% formaldehyde and 1000 µl 100% deionized formamide). The samples were denatured at 60°C for 15 minutes, followed by a 10 minute incubation at 4°C. Twenty microliters of RNA

loading buffer (1mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) was added to each sample, then loaded onto a denaturing gel (1.2% formaldehyde, 1X MOPS, 1% agarose). The samples were electrophoresed in 1X MOPS buffer until the xylene cyanol migrated to the edge of the gel. The gel was rinsed in 0.1% DEPC treated water, and the RNA was transferred to a Zeta Probe nylon membrane (BioRad) by capillary action overnight in a 50mM NaOH solution. The membrane was soaked in 2X SSC (0.3M NaCl, 30 mM sodium citrate), rinsed in dH_2O, then dried at room temperature. The RNA was crosslinked to the membrane by irradiation with UV_{254} for 3 minutes, and stored at room temperature until needed.

2.4.7.2 Prehybridization and Hybridization

The blot was prehybridized in a hybridization oven (VWR Scientific, Model 2710) for 2 hours at 42°C in 30 mls of prehybridization solution (0.5% skim milk powder, 50% deionized formamide, 4% SSPE, 1% SDS and 10 mg/ml herring sperm). Hybridization was performed at 42°C overnight, in 30 mls of hybridization solution (50% deionized formamide, 0.5% skim milk powder, 4% SSPE, 1% SDS, 10% dextran sulphate), containing the radiolabeled probe.

2.4.7.3 Radiolabelling of the probe

The T7 ready prime kit (Pharmacia Biotech) was used to radiolabel the probe. Essentially, the cDNA was electrophoresed on a 1% low melt agarose gel in 1X TBE buffer. The cDNA was trimmed from the gel, removing excess agarose, weighed, and diluted 1:3 with DEPC treated water. Thirty four microliters of the probe were denatured by boiling for 10 minutes, then placed at 37°C for ten minutes. To the probe, 10 µl reagent mix, 5 µl ^{32}dCTP (3000Ci/mmol, New England Nuclear) and 1 µl of T^7 DNA polymerase were added. The reaction was incubated at 37°C for one hour, then 400 µl of column buffer (200 mM NaCl, 10 mM Tris pH 7.5 and 1 mM EDTA, pH 8.0) were added. The radiolabeled probe was boiled for ten minutes, then placed on ice for ten minutes. The entire sample was added to the hybridization buffer.

2.4.7.4 Washing conditions

Following hybridization, the nylon membrane was washed three times to remove the unbound probe. The first wash consisting of 2X SSC and 0.1% SDS, was carried out at 42°C for fifteen minutes. This was followed by a second washing at 42°C for fifteen minutes with 0.5X SSC and 0.1% SDS. The blot was finally washed with 0.1X SSC and 0.1% SDS at 52°C for fifteen minutes. The membrane was air dried, exposed to an X-ray film (Kodak) for one to fourteen days, then developed.

2.4.8 Cloning of the cDNA probe

The PCR-TRAP cloning system was utilized to subclone the probes (GenHunter Corporation, Brookline, MA). The ligation reaction, consisting of 5 µl reamplified cDNA probe, 2 µl pCR-TRAP vector, 1X ligation buffer, and 200 units T4 DNA ligase in a total volume of 20 µl, was incubated overnight at 16°C. Transformation of *E.coli* competent cells was performed by mixing 10 µl of the ligation mixture with 100µl of competent cells, followed by a 45 minute incubation on ice. The cells were heat shocked for two minutes at 42°C, then an aliquot of 400 µL OF LB medium was added. The mixture was incubated for one hour at 37°C. Two hundred microliters of transformed bacteria were plated on LB plates containing 20 µg/ml tetracycline. The plates were incubated in an inverted position overnight at 37°C. The plasmids were verified for inserts by mini-preparation analysis.

2.4.9 Sequencing

Lseq and Rseq primers (AidSeq kit C, GenHunter Corporation, Brookeline, MA) were utilized with the Sequenase Version 2.0 DNA sequencing Kit (United States Biochemicals Co., Cleveland, OH) to sequence the cDNA probes, as described in the instruction manual.

2.4.10 Gene Bank Data analysis

The identity of the probes was determined using computer-based homology search of GenBank data bases using "basic local alignment search tool" (124).

Chapter 3

Transport of [^{3}H] SarCNU and [^{3}H]NE in the SF-295 human glioma cell line

3.1 Summary

SarCNU is a more efficacious anticancer agent compared to BCNU in the treatment of mice bearing subcutaneously implanted SF-295 human glioma xenografts. This suggested that SF-295 cells express uptake$_2$. To assess whether the *in vivo* sensitivity of the SF-295 tumor to SarCNU was associated with the presence of the uptake$_2$ transporter in the established SF-295 cell line, transport of [^{3}H]SarCNU and [^{3}H]NE in the presence of uptake$_2$ inhibitors was investigated *in vitro* at 37°C in suspension. Accumulation of [^{3}H]SarCNU was not decreased by uptake$_2$ inhibitors, and its accumulation at steady state was similar to that of SKI-1 cells. This suggested that uptake$_2$ was not expressed in the SF-295 cell line. Epinephrine has a trophic effect on uptake$_2$ expression. We attempted to upregulate uptake$_2$ in SF-295 cells by treatment with epinephrine. Transport studies of [^{3}H]SarCNU accumulation in SF-295 cells treated with epinephrine indicated that expression of uptake$_2$ was not increased.

3.2 **Introduction**

SarCNU was examined *in vitro* in the National Cancer Institute human tumor panel screen. Glioma cell lines were preferentially sensitive to the antitumor activity of SarCNU as compared to tumors of other origins. The activity of SarCNU versus BCNU, the standard chemotherapeutic agent used in the treatment of gliomas, was compared in the treatment of SF-295 human glioma xenografts in athymic mice. Intravenous, intraperitoneal and oral administrations of optimal concentrations of SarCNU with daily or intermittent dosing schedules cured more animals in comparison to BCNU. BCNU demonstrated a progressive loss of antitumor activity at lower doses while SarCNU retained high antitumor activity even at 66% and at 45% of its optimal dose. These *in vivo* studies support the presence of the uptake$_2$ transporter in the SF-295 cell line (12). Previous studies using the SK-MG-1 human glioma cell line have demonstrated that SarCNU is a substrate for the uptake$_2$ transporter *in vitro* (7-9). It is believed that the presence of this transporter is responsible for the increased accumulation of SarCNU in SK-MG-1 cells, which mediates its enhanced cytotoxic activity (8, 9, 39). The experiments described in this chapter were designed to determine the expression of the uptake$_2$ carrier in SF-295 cells *in vitro*, and to assess whether this correlated with the increased *in vivo* activity of SarCNU as compared to BCNU against the SF-295 tumor xenograft. To evaluate the presence of the uptake$_2$ carrier in the SF-295 cell line, transport

experiments using a modification of the "oil stop methodology" were utilized (7-9, 119). Accumulation of [^{3}H]SarCNU in the presence and absence of uptake$_2$ inhibitors, as well as the accumulation of [^{3}H]NE at 37°C was determined. The results of these studies suggest that the SF-295 cell line does not express the transporter *in vitro*. This implies that the improved activity of SarCNU *in vivo* is not associated with expression of the uptake$_2$ transporter in cultured cells. Epinephrine is believed to have a trophic effect on expression of uptake$_2$ (101, 125). In view of this, we attempted to upregulate the expression of the transporter in SF-295 cells. Epinephrine did not seem to affect the expression of uptake$_2$ at the concentrations attainable.

3.3 **Results**

3.3.1 Time course uptake of [^{3}H]SarCNU and [^{3}H]NE

Figure 3.1 shows the accumulation of 50 μM [^{3}H]SarCNU in SF-295 cells in suspension, at various time points at 37°C. Uptake of [^{3}H]SarCNU was linear until 3 seconds and began to reach equilibrium at 60 seconds. The cell to media accumulation of [^{3}H]SarCNU at steady state was 0.86 (Table 3.2). Figure 3.2 shows the uptake of 25 μM [^{3}H]NE in SF-295 cells at 37°C. Accumulation was linear to 15 seconds. All transport experiments were repeated at least 3 times, and the average values and standard errors were calculated.

3.3.2 Inhibition of [^{3}H]SarCNU and [^{3}H]NE accumulation

The inhibition analysis of [^{3}H]SarCNU transport was performed in SF-295 cells in suspension at 37°C (Table 3.1). Inhibition of [^{3}H]SarCNU uptake with 10 mM epinephrine, 10 mM sarsosinamide, 10 mM SarCNU, 1 μM disprocynium-24 and 10 μM desipramine was carried out at 3 seconds, a time point which represents the initial rate of uptake. Neither uptake$_2$ inhibitors nor the uptake$_1$ inhibitor, desipramine, significantly decreased the accumulation of [^{3}H]SarCNU in the SF-295 cell line, as determined by two-tailed unpaired *t* tests. Inhibition analysis of [^{3}H]NE uptake was conducted. However, due to the low cell to media ratio at three seconds (0.03), inconsistent results were obtained (data not shown).

Figure 3.1

[^{3}H] SarCNU transport in the SF-295 human glioma cell line. The cell to media ratio accumulation of 50 μM [^{3}H]SarCNU was determined in SF-295 cells, in suspension at 37°C at 0, 2, 3, 4, 6, 8, 10, 15, 20, 30, 40 and 60 seconds. Data from 3 individual experiments was averaged.

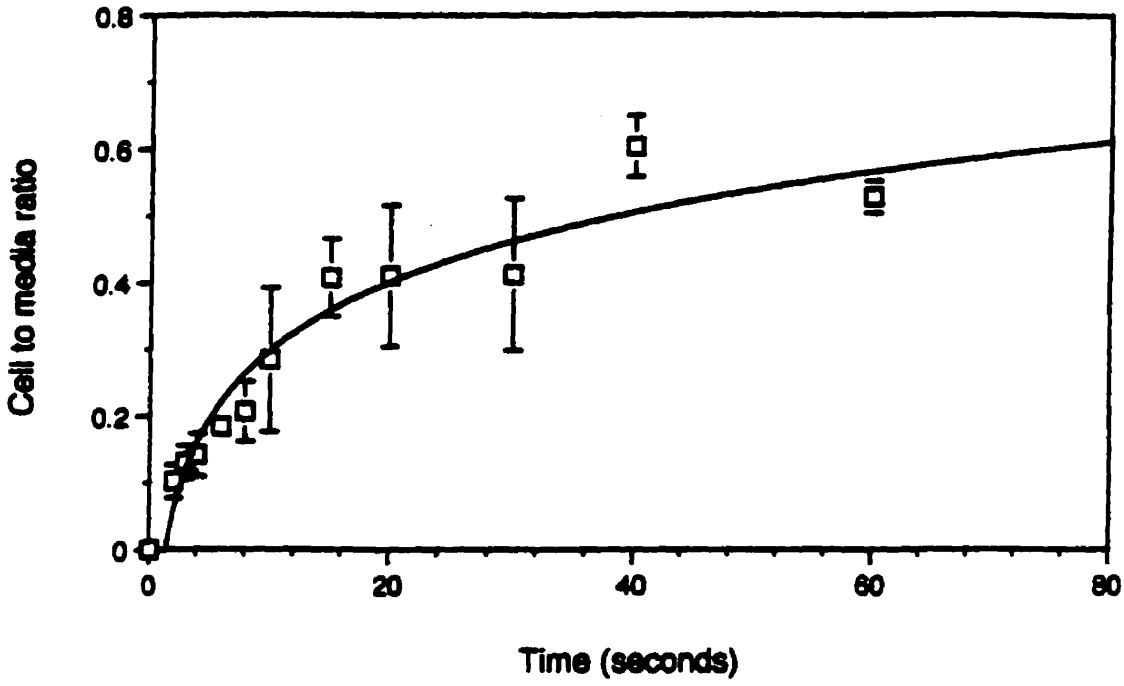

0.8
0.6
0.4
0.2
0
Cell to media ratio
0
20
40
60
80
Time (seconds)

Figure 3.2

[^{3}H]NE uptake profile in the SF-295 human glioma cell line The cell to media ratio accumulation of 25 μM [^{3}H]NE was determined in SF-295 cells in suspension at 37°C at 0, 5, 10 and 15 seconds. Data from 4 individual experiments was averaged.

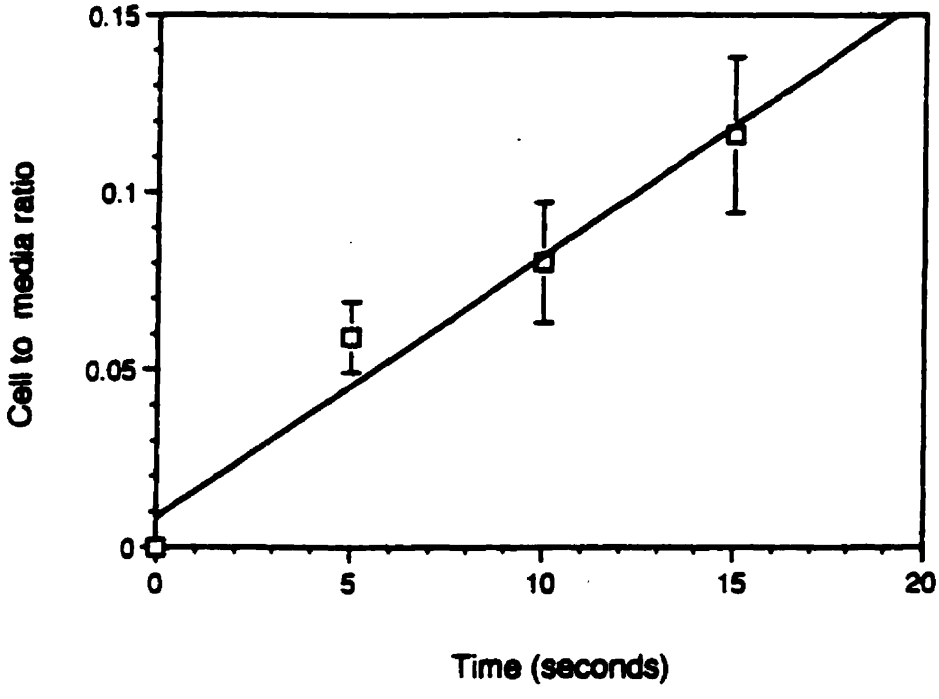

0.15
0.1
0.05
0
Cell to media ratio
0
5
10
15
20
Time (seconds)

Table 3.1

Inhibition studies with [^{3}H]SarCNU in the SF-295 human glioma cell line. SF-295 cells in suspension were examined at 37°C for the 3 second uptakes of 50 μM [^{3}H]SarCNU in the presence or absence of the indicated inhibitors. The mean and standard errors were calculated from values obtained from 5 separate experiments.

Inhibitor	Percent of control uptake[a]	P[b]
Control	100	NS[c]
10 mM Epinephrine	98 ± 9.5	NS
10 mM Sarcosinamide	117 ± 13.6	NS
10 mM SarCNU	134.2 ± 14.7	NS
1 μM Disprocynium-24	107 ± 9.1	NS
10 μM Desipramine	119 ± 6.7	NS

[a] The obtained cell to media ratios were expressed as a percent (average ± standard error) of that obtained from the control.

[b] Significance was determined by the two tailed unpaired *t* test.

[c] Not significantly different from control value.

3.3.3 Effect of epinephrine treatment on uptake$_2$ expression in SF-295 cells

In an attempt to increase the expression of uptake$_2$ *in vitro*, epinephrine was added daily to SF-295 cells in culture. The culture media of SF-295 cells was initially supplemented with a final concentration of 1 μM epinephrine. This concentration was increased weekly, reaching a maximum concentration of 140 μM, for 3 months prior to transport studies. Analysis of [^{3}H]SarCNU uptake utilizing epinephrine treated SF-295 cells indicated that the transport of [^{3}H]SarCNU was similar to that of the non treated cells (figure 3.3).

3.3.4 Intraperitoneal growth of SF-295 cells in athymic mice

Cell to cell interactions or growth factors may be required for uptake$_2$ expression. To test this theory, an *in vivo* study was initiated. SF-295 cells naturally grow as a subcutaneous solid tumor *in vivo*. Under these circumstances, it was not possible to readily investigate the *in vitro* transport of SarCNU after *in vivo* growth of the tumor. We attempted to induce the formation of an SF-295 tumor i.p., which would provide a monocellular suspension of SF-295 tumor cells, that could readily be utilized in transport studies. 10^6 or 10^7 *in vitro* cultured SF-295 cells were injected i.p into BALB/C nude mice. The animals were monitored regularly

by palpitation. Following six weeks, there was no evidence of tumor growth.

3.3.5 Cytotoxicity of SarCNU in relation to steady state accumulation of [^{3}H]SarCNU

We wanted to determine whether the IC_{90} cytotoxicity value of SarCNU correlated with the steady state uptake of [^{3}H]SarCNU at 30 minutes. Steady state accumulation of [^{3}H]SarCNU, in addition to the IC_{90} values of SarCNU cytotoxicity was determined in UW28 and SF-295 glioma cells, and were previously determined in SK-MG-1 and SKI-1 (8) (Table 3.2). SK-MG-1 had the highest steady state accumulation yet had the lowest IC_{90} value for SarCNU cytotoxicity. UW-28 cells, with an intermediate steady state accumulation had the highest IC_{90} value whereas SKI-1 and SF-295 had similarly low accumulations and an intermediate IC_{90} value. The correlation between the cytotoxicity of SarCNU and steady state accumulation, as determined by the student t test was imperfect. This could be secondary to the fact that steady state accumulation of [^{3}H]SarCNU was carried out over 30 minutes, whereas cells in the *in vitro* cytotoxicity assays had continuous exposure to the drug.

Figure 3.3

Uptake of [^{3}H]SarCNU in SF-295 human glioma cells treated with epinephrine. SF-295 cells were treated with epinephrine for 3 months prior to transport studies. The accumulation of 50 μM [^{3}H]SarCNU was determined in these cells, in suspension at 37°C at 0, 2, 3, 4, 6, 8, 10, 15, 20, 30, 40 and 60 seconds. Values from 5 individual experiments were averaged.

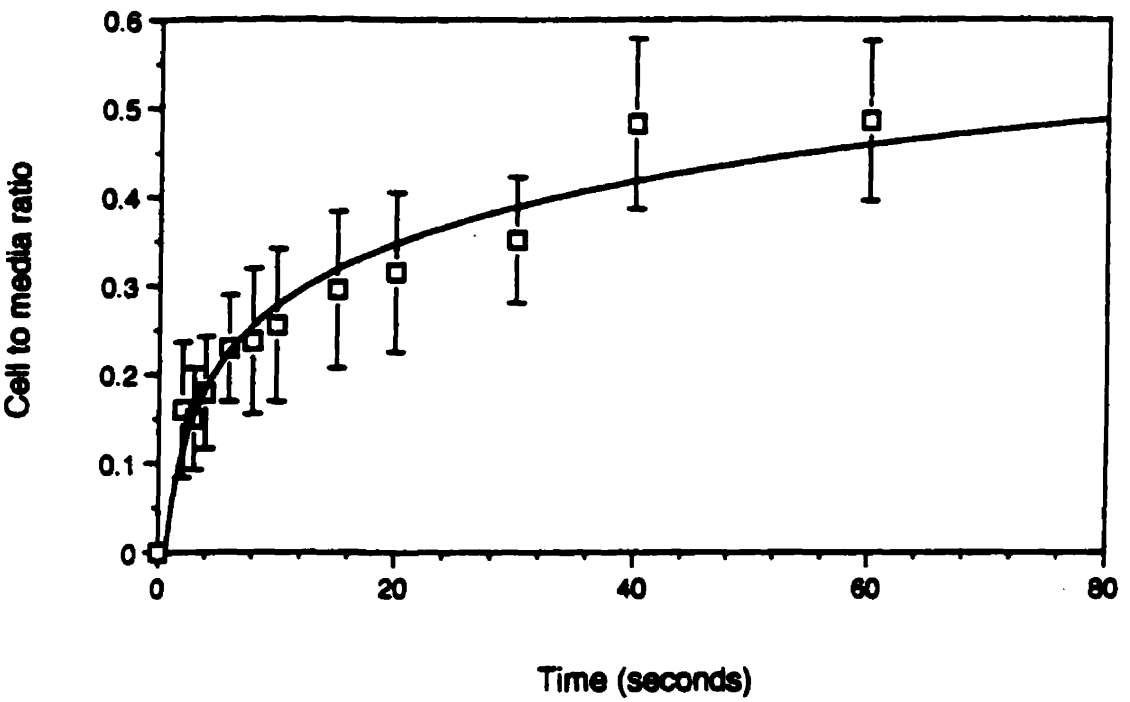

Cell to media ratio
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
Time (seconds)

Table 3.2

Steady state accumulation of [^{3}H]SarCNU and cytotoxicity ratios in glioma cell lines. The steady state accumulation of [^{3}H]SarCNU at 30 minutes and the SarCNU IC_{90} value was determined in UW-28 and SF-295, as described in "Materials and Methods", and was previously determined in SK-MG-1 and SKI-1 cells.

Cell Line	Steady state accumulation[a]	IC_{90} SarCNU (μM)[b]
SK-MG-1	1.28	26.7
UW-28	1.07	62.17
SF-295	0.86	46.5
SKI-1	0.83	44.7

[a] The steady state accumulation at thirty minutes was determined as described in the Materials and Methods section.

[b] The IC_{90} values of SarCNU cytotoxicity were determined as described in the Materials and Methods section.

3.4 Discussion

In the SF-295 cell line, the initial rate of [^{3}H]SarCNU transport at 37°C was similar to that of SKI-1 cells at 37°C (8, 9). However, the initial rate of uptake in SF-295 was decreased when compared to SK-MG-1 cells. Furthermore, the steady state accumulation of [^{3}H]SarCNU in SF-295 cells at 30 minutes was similar to that of SKI-1, 0.86 and 0.83 respectively. Accumulation at steady state in SF-295 cells was decreased by 33% when compared to SK-MG-1 cells, which had a steady state accumulation of 1.28 (8). Taken together, this suggests that SF-295 cells do not express uptake$_2$.

Transport of [^{3}H]SarCNU, in the presence of uptake$_1$ or uptake$_2$ inhibitors was assayed at 3 seconds, a time point which approaches the initial rate of uptake and minimizes the effect of efflux. Desipramine, an uptake$_1$ inhibitor, did not alter [^{3}H]SarCNU transport. More importantly, epinephrine, sarcosinamide, SarCNU and disprocynium-24, inhibitors of the uptake$_2$ carrier, did not decrease the transport of [^{3}H]SarCNU. This suggests that the SF-295 cell line does not express uptake$_2$ *in vitro* and is consistent with passive diffusion as the only mode of entry of SarCNU into these cells. These results were unanticipated following the observed sensitivity of the SF-295 tumor xenograft to the antitumor activity of SarCNU, which highly suggested that these cells expressed the transporter. Inhibition studies of [^{3}H]NE transport were initiated. However, due to the low cell to media ratio at 3 seconds, which was slightly above background values,

consistent results were not obtained (data not shown). Inhibition was not performed at time points greater than three seconds due to metabolism of NE (9).

Previously it was reported that $uptake_2$ can be downregulated *in vivo* following denervation, which was associated with cellular de-differentiation (125). It has been suggested that sympathetic innervation has a trophic influence on the extraneuronal system (101). As cells in tissue culture are not constantly exposed to catecholamines, it is conceivable that $uptake_2$ expression has been downregulated. [^{3}H]SarCNU transport studies in SF-295 cells revealed that the daily addition of epinephrine, in concentrations increasing from 1 μM to 140 μM, did not successfully upregulate $uptake_2$ expression in SF-295 cells as compared to non-treated cells. It was not possible to utilize concentrations of epinephrine above 140μM due to cellular toxicity. Therefore, we propose that a specific *in vivo* microenvironment may be required for $uptake_2$ expression or alternatively, there may be no correlation between the efficacy of SarCNU *in vivo* and the presence of the $uptake_2$ transporter *in vitro*. Other factors such as the increased stability of the drug may be responsible for its increased efficacy against gliomas. Further studies must be performed to determine whether the *in vivo* sensitivity of gliomas correlates with the presence of the transporter. In the future, transport of [^{3}H]SarCNU and [^{3}H]NE with early passage SF-295 cell lines isolated from the SF-295 xenograft will be

examined. This will more closely represent the *in vivo* behavior of uptake$_2$ in SF-295 cells.

We hypothesized that cell lines having a lower IC_{90} value of SarCNU cytotoxicity than that of SKI-1 (44.7 μM) would express the uptake$_2$ transporter. Analysis of 4 human glioma cell lines has suggested that there was no important correlation between the *in vitro* cytotoxicity of SarCNU and *in vitro* transport of [^{3}H]SarCNU at steady state. However, the cytotoxicity assay was a continuous exposure assay. Cytotoxicity assays in which the cells have a one hour exposure to SarCNU and BCNU will be correlated to the steady state accumulation which is performed at 30 - 60 minutes.

Chapter 4

Differential display

4.1 Summary

Our laboratory has accumulated evidence to support the fact that expression of the extraneuronal catecholamine uptake$_2$ transporter in the SK-MG-1 cell line is critical to its response to SarCNU in comparison to SKI-1 cells, which have no detectable transporter. Our long term goal is to clone the uptake$_2$ carrier. To initiate steps in this process, differential display was utilized to detect cDNA fragments overexpressed in SK-MG-1 cells, but diminished in expression in SKI-1 cells. Using differential display, a 180 bp cDNA fragment expressed in SK-MG-1 cells, but lacking in SKI-1 cells, hybridized to an 8 Kb RNA which had a novel sequence and was 62% homologous to an expressed sequence tag of human brain tissue (EST04330). The function and complete sequence of the gene represented by this cDNA is undetermined, and may be a partial sequence of the transporter.

4.2 **Introduction**

Uptake$_2$ appears to play a role in the transport of SarCNU, a promising new experimental CENU analog. Previous studies have shown that the increased accumulation of SarCNU by the uptake$_2$ transporter in SK-MG-1 cells is responsible for its enhanced sensitivity to the drug as compared to SKI-1, a cell line in which the transporter was not detected (7-9). The long term goal of our study was to clone uptake$_2$. The immediate goal of this work was to identify novel differences that exist between SK-MG-1 cells and SKI-1 cells. Novel cDNA sequences expressed in SK-MG-1 could potentially encode the transporter. In order to achieve this, differential display has been utilized (122, 123). This technique can detect altered gene expression between cell lines.

Utilizing differential display, a cDNA probe hybridized to an 8 kB RNA in a Northern analysis, that was detected in SK-MG-1, Caki-1 and FL total RNA preparations which express the transporter, but not in the SKI-1 cell line. The identity and function of the gene represented by the cDNA fragment remains undetermined.

4.3 **Results**

4.3.1 Differential Display

Differential display was utilized to compare SK-MG-1 and SKI-1 cells. Over 70 different primer combinations were available. Of these, the following 32 combinations, $T_{12}MG$, $T_{12}MC$, $T_{12}MT$ and $T_{12}MA$ with AP-1, AP-2, AP-3, AP-4. AP-5. AP-6, AP-7 and AP-8, were utilized. Figure 4.1 shows a partial display using SK-MG-1 and SKI-1 total RNA amplified with the primer combination $T_{12}MC$ and AP-4 (lanes 1 and 2 respectively) in addition to $T_{12}MC$ and AP-5 (lanes 3 and 4 respectively). As seen in figure 4.1, many cDNA fragments are overexpressed in the SK-MG-1 sample. In total, using 32 primer combinations, over 100 cDNA fragments overexpressed in the SK-MG-1 cells were detected. Overexpressed cDNA fragments selected at random were utilized as probes in a northern analysis.

4.3.2 Analysis of probes

The membranes used in the northern analysis contained 60 µg of total RNA from 3 cell lines expressing the transporter (SK-MG-1, FL, and Caki-1) in addition to the negative control cell line, SKI-1. In figure 4.1, arrowheads indicate two cDNA fragments overexpressed in SK-MG-1 cells compared to SKI-1 cells, (hereafter called probe 5 and probe 8), which hybridized to the RNA of all the uptake$_2$ positive cell lines, but to a much lesser extent to the RNA of SKI-1 cells (Figures 4.2 and 4.3). Probe 5 was approximately 180 bp and

hybridized to an RNA species of about 8 Kb (figure 4.2). Probe 8 which was 150 bp long hybridized to a 3 Kb RNA (figure 4.3). The expression of the 8 kb RNA in SK-MG-1 cells was 5 fold greater than the expression in SKI-1 RNA, whereas expression of the 3 kb RNA in SK-MG-1 was 9 fold greater. This was determined by densitometry. Probe 8 and probe 5 were subcloned and sequenced as described in the Materials and Methods section. Using BLAST (124), it was determined that probe 8 had 98% homology with the human glutathione reductase gene whereas probe 5 had 62% homology with an EST clone (EST04330) from human brain tissue (126). Analysis of the sequence of probe 5 using DNA strider version 1.0 in a 3 phase translation mode indicated that there was no open reading frames.

Figure 4.1

Differential Display. Lanes 1 and 2 contain SKI-1 and SK-MG-1 cDNA amplified with $T_{12}MC$ and AP-4 respectively. Lanes 3 and 4 contain SKI-1 and SK-MG-1 cDNA amplified with $T_{12}MC$ and AP-5 respectively. The large arrowhead indicates probe 5, an ~ 180 bp fragment that is overexpressed in SK-MG-1 cells. The small arrowhead indicates probe 8, an ~ 150 bp fragment overexpressed in SK-MG-1.

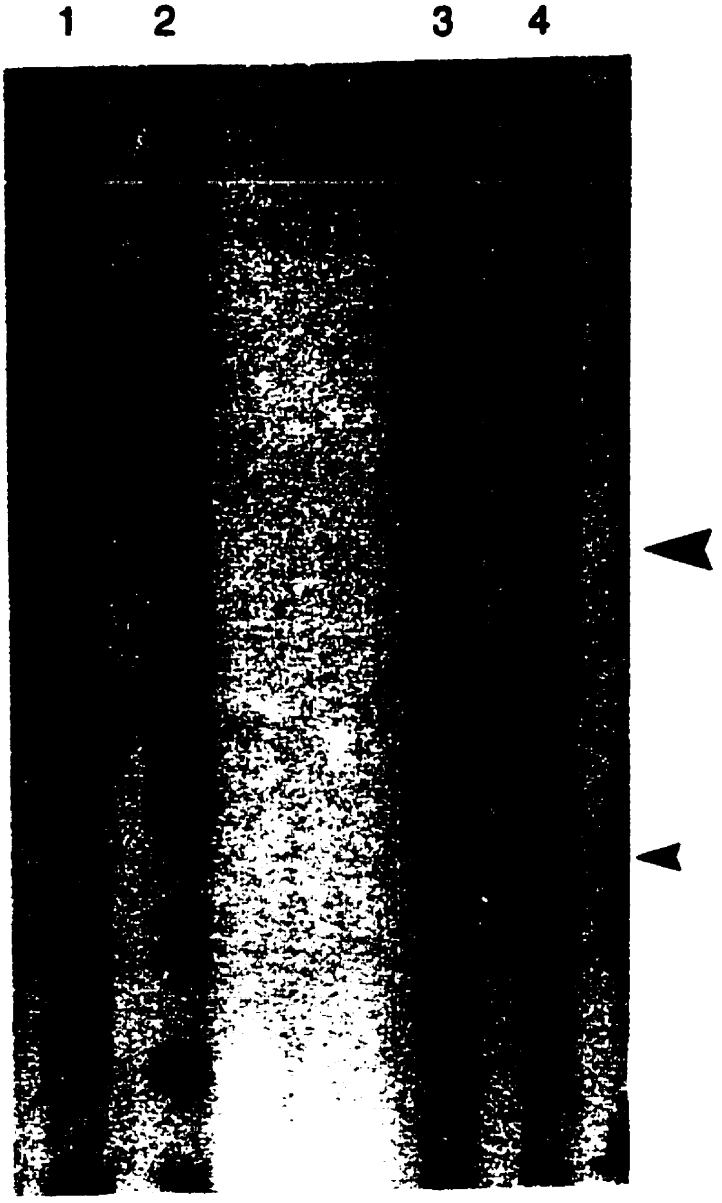

1
2
3
4

Figure 4.2

Northern analysis (A) Probe 5 identified an ~ 8 Kb RNA (arrowhead) expressed in Caki-1 (lane 1), FL (lane 2) and SK-MG-1 (lane 3) cell lines, but not in SKI-1 (lane 4). β-actin indicated that the total RNA was similarly loaded in all lanes (figure 4.3).

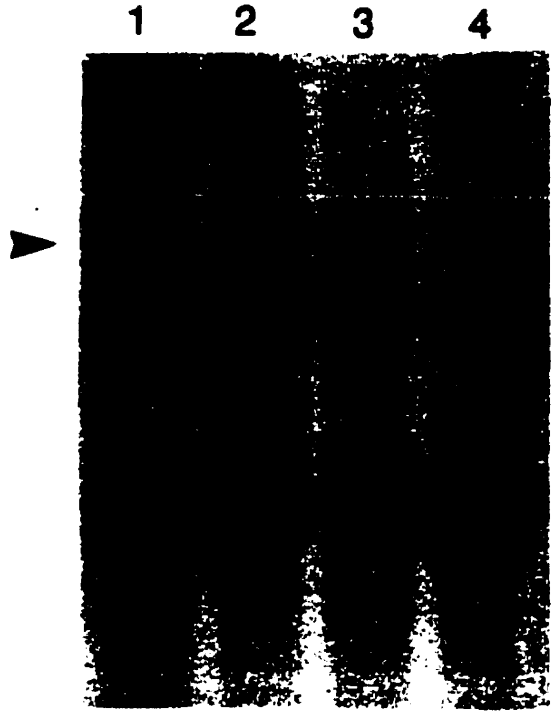

1
2
3
4

Figure 4.3

Northern analysis (A) Probe 8 hybridized to a 3 Kb RNA that was expressed in Caki-1 (lane 1), FL (lane 2) and SK-MG-1 (lane 3) cell lines, but to a lesser extent to the RNA of SKI-1 cells (lane 4). (B) β-actin indicated that the total RNA was similarly loaded in all lanes.

1 2 3 4

A. 

B. 

4.4 **Discussion**

The enhanced sensitivity of glioma cells to SarCNU has been associated with the selective accumulation of the drug by the extraneuronal $uptake_2$ transporter (7-9). Therefore, we were interested in cloning $uptake_2$. Technical difficulties have impaired the cloning process. The low affinity and nonconcentrative accumulation of catecholamines, in addition to the substantial amount of nonfacilitated diffusion, suggests that the approach utilized to clone $uptake_1$ was unlikely to be successful in the cloning of $uptake_2$. At this time, neither an antibody nor a probe has been developed against $uptake_2$. Therefore, we have utilized differential display to initiate steps in the cloning process.

The finding that SKI-1 cells have no detectable $uptake_2$ transporter may be due to the lack of sensitivity in the method utilized to describe the transporter in SKI-1 cells. Therefore, the possibility that SKI-1 cells express $uptake_2$, albeit at much lower concentrations than SK-MG-1 cells, is not excluded. Therefore cDNA fragments in the differential display expressed in SK-MG-1, but expressed to a much lesser extent in SKI-1 samples were examined, in addition to cDNA fragments that were completely lacking in SKI-1. In the northern analysis, many probes did not produce a signal and were likely to be artifacts from the display. Other probes did not produce the appropriate hybridization pattern in the northern analysis, meaning that they did not hybridize to the RNA of Caki-1, FL and SK-MG-1 simultaneously. Two probes,

probe 5 and probe 8, represented real differences between SKI-1 and SK-MG-1 cells. These probes hybridized to the RNA of the uptake$_2$ positive cell lines, but to a lesser extent to the RNA of SKI-1. Probe 8 was identified as glutathione reductase, an enzyme involved in the biosynthesis of glutathione. This result was consistent with the finding that SKI-1 cells expressed 8 fold less GSH than SK-MG-1 cells. Probe 5 represented a novel sequence, having 62% homology with the EST04330 clone previously identified in human brain tissue. This probe could potentially be a partial sequence of the uptake$_2$ transporter (126). The RNA species represented by probe 5 is of exceptional interest as it is expressed in 3 cell lines with detectable extraneuronal carrier but is much less expressed in SKI-1 and has not been previously cloned. A large RNA species is consistent with a large transmembrane protein. However, an 8 Kb RNA may have a smaller open reading frame (<2 kb). At this time, the significance of this finding is unclear, as the actual size of the transporter in unknown. Further characterization of this sequence must be carried out to determine its identity and function.

Chapter 5

General discussion

5.1 **Discussion**

In this thesis, we set out to determine whether:

1- The increased efficacy of SarCNU versus BCNU in the treatment of subcutaneously implanted SF-295 glioma xenografts was indicative of the presence of the uptake$_2$ transporter in cultured SF-295 cells.

2- Novel differences expressed in the SarCNU sensitive cell line SK-MG-1, but with diminished expression in the relatively resistant SKI-1 cells, could be identified.

In view of our first objective, our work suggested that the extraneuronal uptake$_2$ transporter was not expressed in the SF-295 cell line *in vitro*. Influx of [^{3}H]SarCNU in SF-295 cells was similar to that of SKI-1 yet significantly decreased when compared to SK-MG-1 which expresses the transporter (7-9). Influx of [^{3}H]SarCNU was not decreased in the presence of excess concentrations of uptake$_2$ inhibitors, implying that SarCNU enters SF-295 cells only by passive diffusion. The finding that SKI-1 and SF-295 cells do not express a detectable transporter may be because they lack the uptake$_2$ transport system or alternatively, the levels of uptake$_2$ expression may be extremely low. This makes it difficult to differentiate between low uptake$_2$ expression and absence of the protein, using [^{3}H]SarCNU accumulation as an

indicator of the transporter, as the km of SarCNU transport by uptake$_2$ is in the mM range in SK-MG-1 cells. It has been demonstrated that SKI-1 cells have a carrier for sarcosinamide. However its affinity for sarcosinamide was significantly reduced as compared to the transporter in SK-MG-1 cells. A recent report by Russ *et al* indicated that various primary culture human gliomas express a saturable extraneuronal catecholamine transport system. [^{3}H]MPP+, which has a very high affinity in the nM range for the uptake transporter, was utilized to characterize these cell lines (108). This suggests that MPP+ may be able to detect a smaller number of transporters in glioma cells than either [^{3}H]SarCNU or [^{3}H]NE. In addition, the primary cultures utilized by Russ *et al* were early passage cell lines. This may more closely represent the *in vivo* expression of uptake$_2$. In contrast, the SF-295 cell line utilized in our studies has been in culture for a longer time.

It has been suggested that the uptake$_2$ transporter is regulated by adrenergic innervation (101, 125). During the establishment of a cell line, the effects of adrenergic innervation are lost, and cell lines could downregulate or lose expression of the extraneuronal uptake$_2$ system. For this reason, it would be of primary interest to conduct [^{3}H]SarCNU, [^{3}H]NE, and [^{3}H]MPP+ transport studies on early passage SF-295 cells established from tumor xenografts which demonstrated an increased sensitivity to SarCNU compared to BCNU. Alternatively, the enhanced sensitivity of SarCNU in the

treatment of the SF-295 human tumor xenografts in athymic mice may be a consequence of the increased chemical half life of SarCNU versus that of BCNU. At physiological conditions, the chemical half life of SarCNU was 6 fold greater when compared to BCNU. This is a consequence of the sarcosinamide side group attached at the N3 position of SarCNU, which alters its mechanism of degradation. This is in contrast to BCNU, which has a short chemical half life and is rapidly degraded *in vivo*.

As concerns our second objective, we identified a novel cDNA fragment by differential display that could potentially be a partial sequence of the uptake$_2$ transporter. The identity of the cDNA that hybridized to the 8 Kb RNA remains undetermined. Ultimately, the complete cDNA sequence must be obtained by screening an SK-MG-1 cDNA library, followed by transfection experiments into a cell line that lacks the transporter. Functional transport studies with [^{3}H]SarCNU, [^{3}H]NE and [^{3}H]MPP+ in the transfected and control cell lines will be necessary to determine whether this sequence encodes uptake$_2$.

The finding that glutathione reductase, by differential display, is more highly expressed in SK-MG-1 as compared to SKI-1 cells demonstrates the capability of this technique in detecting differences between the cell lines. The drawbacks of this method involve the large amount of differences detected between the cell lines, making it tedious to locate one specific gene. Ideally, it would have been preferable to

chose a cell line in which expression of the transporter has been altered, or could be regulated. We attempted to upregulate the transporter in SF-295 cells by epinephrine treatment. However, epinephrine did not alter the expression of uptake$_2$.

Concluding Remarks

Characterization of the uptake$_2$ transporter is significant as it may be involved in the entry of SarCNU into selected tumor cells. This mechanism of transport may account for the increased effectiveness of SarCNU versus BCNU against gliomas *in vivo*. Once the carrier is characterized, *in situ* hybridization and immunohistochemistry studies, in clinical samples would determine whether a correlation exists between the expression of uptake$_2$ *in vivo* and SarCNU efficacy. Identification of tumors expressing the uptake$_2$ carrier may select for tumors with enhanced susceptibility to SarCNU. Also, a detailed knowledge of the pathways of inactivation of monoamines in the CNS is fundamental to the understanding of normal brain function, and for the development of drugs which may act through the modulation of extracellular levels of monoamines in the CNS.

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