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REVIEW

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SarCNU (2-chloroethyl-3-sarcosinamide-1-nitrosourea): a novel analogue of chloroethylnitrosourea that is transported by the catecholamine uptake$_2$ carrier, which mediates increased cytotoxicity

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Introduction

High-grade gliomas are inevitably fatal, with an average survival of 6–12 months following surgery [16]. One of the most effective chemotherapeutic agents for malignant gliomas is bis-chloroethylnitrosourea (BCNU) [17]. The chloroethylnitrosoureas result in dose limiting toxicity of delayed cumulative myelosuppression [2]. In spite of its demonstrated activity in malignant gliomas, the combination of BCNU and radiotherapy is not clearly superior to radiotherapy alone [18]. The development of novel agents that are more active against gliomas would be very useful. The clinically available chloroethylnitrosoureas which include BCNU, CCNU and methyl CCNU all enter cells via passive diffusion [1]. In addition, all of these agents decompose intracellularly to form organic isocyanates which carbamoylate the epsilon amino groups of lysine, the hydroxyl groups of serine, and α-amino groups in proteins but do not significantly attack RNA or DNA [19]. Moreover, these compounds form chloroethyl diazohydroxide moieties which are alkylating agents that attack RNA, DNA and protein [5].

SarCNU (2-chloroethyl-3-sarcosinamide-1-nitrosourea) is a novel analogue of the chloroethylnitrosoureas, which does not form an organic isocyanate because the N-3 position is blocked with a methyl group (see Fig. 1) [15]. Thus, the decomposition of this compound is unique in this class of agents. The carrier group which is a methylglycinamide (sarcosinamide) is an amino acid amide. The compound was originally synthesized with the impression that the carrier group would allow for transport by the amino acid transporters. SarCNU was initially screened in our laboratory in the human tumour cloning assay. The initial results with four high grade gliomas revealed that SarCNU was significantly more active than BCNU at equimolar concentrations. In addition, utilising human bone marrow specimens from normal volunteers in the GM-CFU-C assay, SarCNU was approximately sevenfold less myelotoxic than BCNU in this assay [9]. This suggested that SarCNU had an improved therapeutic index as compared to BCNU in the treatment of gliomas. Furthermore, another laboratory confirmed our results that SarCNU was more toxic to human gliomas in the human tumour cloning assay (Arlene Mulne, Children's Medical Center Dallas, Texas, personal communication).

Previously, it has been demonstrated that drugs, utilised in the in vitro human tumour cloning assay, that reduce colony growth to ≤30% of control, correlate with an excellent chance of producing a response in those patients from which the tumours have been obtained [3]. We determined the concentration of BCNU or SarCNU which resulted in a suppression of growth to ≤30% of the control values with 13 primary glioma specimens. At equimolar concentrations, SarCNU reduced colony growth to ≤30% of control with 11 of 13 glioma specimens, whereas BCNU produced an equivalent result in only 6 of 13 glioma specimens [12].

Since SarCNU has not been used clinically, it is not known what peak plasma concentrations are obtainable with this drug. It is possible to estimate the peak plasma concentration based on the LD_{50} dose in normal BDF-1 mice [11]. SarCNU is approximately tenfold less toxic to mice than BCNU. This correlates with the increased stability of SarCNU [15]. The estimated peak plasma concentration of SarCNU utilising this formula is 68 μM as compared to the actual plasma concentration of approximately 9 μM (calculated value is 10.3 μM) for BCNU. When we reanalysed the data, utilising the peak plasma concentration of each drug as the maximum obtainable concentration, SarCNU reduced colony growth to ≤30% of

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Table 1 Properties of 2-chloroethly-3-sarcosinamide-1-nitrosourea (*SarCNU*) versus bis-chloroethylnitrosourea (*BCNU*). [*IC_{50} of CFU-C* the concentration that reduces colony growth of normal human bone marrow precursor cells to 50% of control values, *PPC* the predicted (SarCNU) or achievable (BCNU) peak plasma concentration, *HTCA (ED_{30}) activity at PPC* the number of primary gliomas or glioma cell lines that are sensitive (reduce growth to ≤30% of control) at the PPC]

Property	SarCNU	BCNU	Fold difference
Molecular weight	222.5	214	
Chemical half-life [13, 15]	333 min	57 min	6
LD_{50} [11, 15]	392 mg/kg	35 mg/kg	11
IC_{50} of CFU-C [9]	36 μM	5 μM	7
PPC [12]	68 μM	9 μM	7.5
HTCA (ED_{30}) activity at PPC			
Primary gliomas [12]	10/13	1/13	
Glioma cell lines [12]	1/3	0/3	

Fig. 1 The chemical decomposition of bis-chloroethylnitrosourea (*BCNU*) and 2-chloroethyl-3-sarcosinamide-1-nitrosourea (*SarCNU*). N^* indicates the N-1 position and N# or N# indicates the N-3 position in the chloroethylnitrosourea molecule. Both BCNU and SarCNU decompose to form a chloroethyl diazohydroxide moiety but only BCNU forms an organic isocyanate

control in 10 of 13 glioma specimens, while BCNU was only active with 1 of 13 glioma specimens. In addition, we examined three human glioma cell lines in the human tumour cloning assay. One of the 3 glioma cell lines was sensitive to SarCNU at its achievable peak plasma concentration, while none of the three cell lines was sensitive to BCNU [12]. The activity of these drugs was next compared in the intracranial glioblastoma (human glioma cell line U-251) athymic mouse model. BCNU at a maximally tolerated dose resulted in 60% long-term survivors, while SarCNU at both its maximally tolerated dose and 60% of that dose resulted in 90–100% long-term survivors. Thus, SarCNU appeared more active in this model than BCNU and, in addition, was 12-fold less toxic [4]. A summary of the comparative properties of SarCNU versus BCNU is shown in Table 1.

In order to examine the uptake and transport of SarCNU in the sensitive human glioma cell line, SK-MG-1, [^{3}H]sarcosinamide was synthesized. Utilising sarcosinamide, the carrier group of SarCNU, we demonstrated that there was saturable uptake of this compound in the SK-MG-1 cells [13]. Furthermore, the uptake was temperature dependent, sodium independent, and inhibited by unlabelled excess sarcosinamide or SarCNU [14]. Sarcosinamide uptake, over a 200-fold range of concentrations, followed Michaelis-Menten kinetics with a K_m of 0.284 mM and a V_{max} of 0.154 nmol/10^6 cells per min. Dixon plot analysis demonstrated that there was competitive inhibition of sarcosinamide uptake by SarCNU with a K_i of 3.26 mM. Utilising an insensitive colourimetric assay and 1 mM drug concentrations, the steady state intracellular concentration of SarCNU was found to be significantly higher (cell:medium ratio of 1.03) as compared to that of BCNU (cell:medium ratio of 0.52). These findings indicated that SarCNU and sarcosinamide appeared to share the same carrier for uptake in SK-MG-1 cells. We tested a number of potential transport systems by using excess unlabelled compounds such as amino acids, choline and glucosamine. None of these compounds appeared to be the native substrate for this carrier system. However, epinephrine did reduce the uptake of sarcosinamide in a fashion similar to that of sarcosinamide itself. Dixon plot analysis demonstrated that epinephrine inhibited the uptake of sarcosinamide competitively with a K_i of 0.26 mM, which is similar to the K_m of 0.27 mM of epinephrine in this cell line [14]. These results indicate that sarcosinamide is a substrate for the catecholamine transporter. In summary, it appeared that the increased accumulation of SarCNU in these cells was secondary to the carrier-mediated transport of SarCNU via the catecholamine transporter.

A comparison of the structures of BCNU, SarCNU, sarcosinamide, epinephrine and norepinephrine is shown in Fig. 2. Epinephrine, sarcosinamide and SarCNU share a common elemental structure involving an N-methyl group plus an ethyl group with a common oxygen site in altered oxidative states (hydroxyl group in epinephrine versus a carbonyl group in sarcosinamide and SarCNU). In order to determine precisely the transport of SarCNU in the SK-MG-1 cells, [^{3}H]SarCNU was obtained. The uptake of [^{3}H]SarCNU was found to be temperature dependent with influx being linear only to 4 s at 37 °C. Equilibrium was reached after 1 min at 22 °C and 37 °C, with accumulation slightly above unity. The initial rate of uptake was significantly more rapid at 37 °C as compared to that at 22 °C. Epinephrine was able significantly to inhibit the uptake of [^{3}H]SarCNU at 37 °C. Again, several amino acids were unable to inhibit the uptake of SarCNU. The initial rate of SarCNU influx was shown to be mediated by both facilitated and non-facilitated diffusion. The component of SarCNU influx via facilitated diffusion obeyed Michaelis-

Fig. 2 The chemical structures of chloroethylnitrosoureas and related compounds. Epinephrine, sarcosinamide and SarCNU share a common elemental structure involving an N-methyl group plus an ethyl group with a common oxygen site in altered oxidative states (hydroxyl group in epinephrine versus a carbonyl group in sarcosinamide and SarCNU)

Menten kinetics over a 200-fold range of concentrations with a K_m of 2.4 mM and a V_{max} of 240 pmol/μl of intracellular water/s. Dixon plot analysis corrected for non-facilitated diffusion of SarCNU revealed that epinephrine inhibited the uptake of SarCNU competitively with a K_i of 163 μM [7]. In addition, Dixon plot analysis of norepinephrine inhibition of uptake of SarCNU revealed competitive inhibition with a K_i of approximately 300 μM. These findings are consistent with the hypothesis that the facilitated component of [^{3}H]SarCNU uptake is via the catecholamine uptake$_2$ carrier system. SarCNU is thus the first chloroethylnitrosourea that has been demonstrated to have carrier-mediated uptake.

In order to demonstrate that the transport of [^{3}H]SarCNU is important in the cytotoxicity of this agent, the transport kinetics of [^{3}H]SarCNU were next examined in the sensitive SK-MG-1 glioma cells in comparison to the resistant SKI-1 glioma cells. At 37 °C, uptake of 50 μM [^{3}H]SarCNU was linear to 4 s in both cell lines, with uptake being significantly faster in SK-MG-1 cells as compared to SKI-1 cells under initial rate conditions [8]. However, there was no significant difference in the rate of influx at 22 °C between both cell lines. Equilibrium was approached at 1 min in both cell lines at 22 °C and 37 °C. At 37 °C, steady state accumulation of SarCNU, at 30 min, was reduced significantly in SKI-1 cells compared to SK-MG-1 cells, although accumulation was similar at 22 °C in both cell lines. In SK-MG-1 cells, uptake of [^{3}H]SarCNU at 37 °C was found to be saturable, while uptake in SKI-1 cells was not saturable over a 1000-fold range of concentrations. Analysis of efflux in both cell lines revealed that the efflux was equivalent in both cell lines but that efflux was more rapid at 37 °C compared to that at 22 °C. There was no difference in metabolism of SarCNU after a 60 min incubation in either cell line. SKI-1 cells compared with SK-MG-1 cells were threefold more resistant to SarCNU at 37 °C but only twofold more resistant at 22 °C, a temperature at which SarCNU accumulation was similar in both cell lines. The twofold resistance at 22 °C was similar to that of BCNU at 37 °C and 22 °C. These findings indicate that the increased cytotoxicity in SK-MG-1 cells is associated with a greater accumulation of SarCNU via an epinephrine-sensitive carrier that is not detected in SKI-1 cells [8]. Therefore, the uptake of SarCNU via the catecholamine uptake$_2$ carrier may be responsible for its increased cytotoxicity in glioma cells compared with BCNU and other clinically available chloroethylnitrosoureas that enter cells via passive diffusion.

The catecholamine uptake$_2$ transporter, which is inhibited by O-methylated catecholamines, corticosteroids and beta-haloalkylamines, is clearly different from uptake$_1$ and exists in various tissues such as myocardium, salivary glands and vascular smooth muscle [6]. Recently, the presence of the catecholamine uptake$_2$ carrier has been described in a human renal cell carcinoma cell line, suggesting that other tumours may be selectively sensitive to SarCNU [10]. SarCNU is currently undergoing extensive toxicology studies at the National Cancer Institute with a view to potential clinical trials.

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