Cancer Chemother Pharmacol (1992) 31: 146–150

Cancer Chemotherapy and Pharmacology

Lack of evidence for a high-affinity sarcosinamide carrier or a catecholamine carrier in Calu-1 lung-cancer cells, HT-29 colon-cancer cells, and DHF fibroblasts

Areti Malapetsa, Jonathan L. Bramson, Adrian J. Noë, Lawrence C. Panasci

Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, 3755 Cote Ste-Catherine Road, Montreal, Quebec, Canada H3T 1E2

Received 10 March 1992/Accepted 3 August 1992

Summary. We have previously demonstrated that uptake of the amino acid amide sarcosinamide by the glioma cell line SK-MG-1 occurs via the catecholamine carrier that accomodates epinephrine (K_m = 0.284 mM; V_{max} = 0.154 nmol/10^6 cells/min). Sarcosinamide chloroethylnitrosourea (SarCNU), a new anticancer agent that exerts increased in vitro antitumor activity against gliomas as compared with BCNU (bis-chloroethylnitrosourea), the standard agent of choice, competitively inhibits sarcosinamide uptake by SK-MG-1 cells [inhibition constant (K_i) = 3.26 mM]. Using radiolabeled *N*-[^{3}H]-sarcosinamide, we determined the transport of sarcosinamide in HT-29 colon-cancer cells, in Calu-1 lung-cancer cells, and in normal foreskin DHF fibroblasts. Sarcosinamide transport was linear for up to 1 min at 22° C. In HT-29 cells and DHF fibroblasts, the uptake of sarcosinamide followed Michaelis-Menten kinetics of carrier-mediated transport. In HT-29 cells the Michaelis constant (K_m) was 2.76 ± 0.1 mM and the maximal velocity (V_{max}) was 2.03 ± 0.1 nmol/10^6 cells/min, whereas in DHF fibroblasts the respective values were 6.58 ± 3.90 mM and 12.08 ± 8.20 nmol/10^6 cells/min. In these two cell lines, neither epinephrine nor leucine significantly reduced sarcosinamide transport. In Calu-1 cells there was no evidence of carrier-mediated transport of either sarcosinamide or epinephrine. These nonglial cell lines lack a high-affinity catecholamine carrier. The increased cytotoxicity of SarCNU in gliomas may correlate with the presence of a high-affinity catecholamine carrier.

Introduction

Chloroethylnitrosoureas (CENUs) are an important class of antitumor agents that exert activity against a variety of human cancers, including certain types of lung cancers and gliomas [3]. The mechanism of cytotoxicity of CENUs is assumed to be secondary to the alkylation of DNA by the chloroethyl moiety. The covalent interstrand cross-links formed between complementary strands of DNA interfere with the replication and transcription of DNA, presumably resulting in cell death. It has been shown that CENU-induced cytotoxicity correlates with the ability to form DNA interstrand cross-links, but there are indications that other DNA lesions may be implicated in the process [4, 5, 7].

CENUs are the most effective clinically available chemotherapeutic agents for the treatment of malignant gliomas. However, only 30% of patients with malignant gliomas sustain a partial response to CENU therapy [15]. Thus, resistance to CENUs is a common problem that may be due to increased DNA repair [2, 12], CENU inactivation via metabolism [13], and/or decreased drug accumulation [10].

Sarcosinamide chloroethylnitrosourea (SarCNU) is a new CENU that contains an amide of methylglycine, sarcosinamide [14]. SarCNU has been shown to be more active than bis-chloroethylnitrosourea (BCNU), the standard chemotherapeutic agent, against primary and established glioma cells in vitro [6, 9]. In addition, SarCNU is less myelotoxic than BCNU in vitro [6]. The presence of the sarcosinamide moiety in SarCNU suggests that its transport into cells may occur via a carrier-mediated mechanism, in contrast to the clinically available CENUs, which enter cells by passive diffusion [1].

The transport of sarcosinamide into SK-MG-1 gliomas is mediated by the uptake-2, energy-independent, sodium-independent catecholamine carrier that accommodates epinephrine. The Michaelis constant (K_m) of sarcosinamide uptake by SK-MG-1 cells is 0.284 mM [maximal velocity (V_{max}) = 0.154 nmol/10^6 cells/min), which is similar to that of epinephrine (K_m = 0.270 mM). SarCNU competitively inhibits sarcosinamide transport, its inhibition constant (K_i) being 3.26 mM. Therefore, in SK-MG-1 cells, the 2-fold greater intracellular concentration of SarCNU as compared with BCNU may be related to differential uptake [11]. The SKI-1 glioma cell line, which exhibits 20-fold resistance to SarCNU as compared with the SK-MG-1 glioma cell line, had altered kinetic constants of sarcosinamide transport (K_m = 1.5 mM, V_{max} = 0.66 nmol/10^6

Correspondence to: L. C. Panasci, Lady Davis Institute for Medical Research

cells/min; SarCNU K_i = 17.5 mM) but a similar time course of sarcosinamide uptake in comparison with SK-MG-1 cells. This was associated with a decreased accumulation of SarCNU in SKI-1 cells [10].

The specificity of sarcosinamide transport and its relationship to epinephrine in other tissues was examined in the present study. We determined the kinetic parameters of sarcosinamide transport into normal human fibroblasts, a human lung-cancer cell line, and a human colon-cancer cell line.

Materials and methods

Materials. *N*-[^{3}H]-Sarcosinamide hydrochloride (1.05 Ci/mmol) was custom-synthesized by Amersham Laboratories (Buckinghamshire, England) using a catalyzed exchange reaction with high-specific-activity tritiated water. The radiochemical purity was 96% as revealed by thin-layer chromatography carried out on cellulose in butan-1-ol: water: acetic acid (12:5:3, by vol.) or ethanol: 0.88 ammonia: water (80:4:16, by vol.), producing R_f values of 0.34 and 0.68, respectively. L-[*N*-methyl-^{3}H]-epinephrine was purchased from New England Nuclear (Dupont Canada, Pointe Claire, Quebec). Sarcosinamide HCl, (±)-epinephrine HCl, L-leucine, glycylglycine HCl, and 5-sulfosalicylic acid were purchased from Sigma Chemical Company (St. Louis, Mo.). Sodium acetate, sodium chloride, D-glucose (anhydrous), and chloroform were purchased from Fisher Scientific Company. McCoy's modified medium 5A, RPMI 1640 medium, minimum essential medium (MEM), MEM nonessential amino acids (NEAA), MEM sodium pyruvate solution, fetal calf serum (FCS), Dulbecco's phosphate-buffered saline (PBS), trypsin-ethylenediaminetetraacetic acid (EDTA), and phenol red solution were obtained from Grand Island Biological Co. (Grand Island, N.Y.). Bovine serum albumin (BSA, fraction V) was purchased from Miles Laboratories.

Cells. The HT-29 cells were established from a primary colon adenocarcinoma; the Calu-1 cells, from a single epidermoid lung-carcinoma specimen; and the DHF fibroblasts, from the foreskin of a healthy man. The human-cancer cell lines HT-29 and Calu-1 were kindly supplied by Dr. M. Pollak, and the DHF fibroblasts were a generous gift from Dr. R. Germinario (both from Lady Davis Institute for Medical Research, Montreal, Quebec). All three cell lines were grown and maintained as monolayers of cells in a humidified atmosphere containing 5% CO_2 at 37° C. The HT-29 cells were maintained in RPMI 1640 medium supplemented with 5% FCS and 4 µg gentamycin/ml (Schering, Pointe Claire, Quebec). The Calu-1 cells were maintained in McCoy's modified medium 5A supplemented with 10% FCS and 4 µg gentamycin/ml, and the DHF fibroblasts were maintained in MEM supplemented with 10% FCS, 1.0% NEAA, 0.9% sodium pyruvate, and 4 µg gentamycin/ml.

Transport experiments. The transport of *N*-[^{3}H]-sarcosinamide was conducted on confluent monolayers of HT-29 cells (2.5 × 10^6 cells/35-mm plate), Calu-1 cells (1.0 × 10^6 cells/35-mm plate), and DHF fibroblasts (2.5 × 10^5 cells/35-mm plate) at 22° C using a method described by Ronquist et al. [8] and previously applied in this laboratory [11]. The media were removed by aspiration, and the cells were washed once with 2 ml phosphate-buffered saline (pH 7.4) supplemented with 0.1% BSA (PBS). The cells were preincubated for 15–30 min in 2 ml PAG (phosphate-buffered saline supplemented with 0.7% BSA, 0.25% D-glucose, and 0.001% phenol red; pH 7.4) at 22° C, the PAG was aspirated, and transport was initiated by the addition of 1 ml *N*-[^{3}H]-sarcosinamide alone or combined with different concentrations of osmotically adjusted physiological inhibitors in PAG. At desired time points, the radiolabel was rapidly aspirated and the cells were rinsed five times with ice-cold PBS supplemented with 48 mM glycylglycine. Glycylglycine was included in the rinsing buffer because it removes radiolabel bound to the plastic surface of the petri dish. This step was completed in about 8 s, and the cells remained attached to the plates after this procedure. The cells were then solubilized with 2 ml 3% 5-sulfosalicylic acid at 60° C for 10 min and the cell extracts were transferred to borosilicate tubes and heated to 100° C for 5 min. The cellular debris was pelleted by centrifugation at 300 *g* for 10 min, and 800 µl of the supernatant was removed and counted in 10 ml scintiverse.

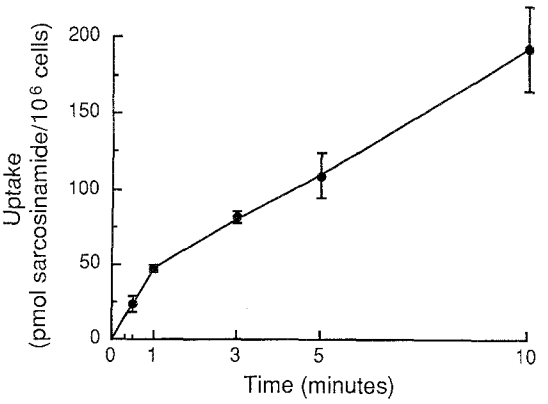

Fig. 1. Time course of the uptake of 0.05 mM *N*-[^{3}H]-sarcosinamide by HT-29 monolayers at 22° C. The uptake is expressed as picomoles of tritiated sarcosinamide per 10^6 cells as described in Materials and methods. Points represent mean values for 3 separate experiments, respectively; bars indicate the SE

The number of cells per 35-mm plate was determined by detaching the cells from the surface of the plate with 1 ml trypsin-EDTA for 5 min and counting the resultant cells using a model F_N Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). Four to six plates were counted, with the average corresponding to the cell number per plate. The standard error of the mean of the cell counts obtained by this method is less than or equal to 5%. Background radioactivity was determined at time zero at 22° C and the resultant background value was subtracted from each determination. The uptake of sarcosinamide was expressed in picomoles per 10^6 cells. Determination of the transport of [^{3}H]-epinephrine was conducted in a fashion similar to that outlined above for sarcosinamide. The uptake of [^{3}H]-epinephrine was determined at 22° C in PAG.

Metabolism of [^{3}H]-sarcosinamide. The possibility of intracellular metabolism of [^{3}H]-sarcosinamide was examined by thin-layer chromatography as previously described [11].

Results

Time course of uptake of N-[^{3}H]-sarcosinamide

The time course of the uptake of 0.05 mM sarcosinamide by monolayers of HT-29 cells at 22° C is shown in Fig. 1. In HT-29 cells, the uptake after 1 min was 47.1 ± 2.2 pmol/10^6 cells. No evidence of metabolism of sarcosinamide was observed after 10 min in the HT-29 cells; this finding was similar to results previously obtained in SK-MG-1 and SKI-1 cells [10, 11]. In DHF fibroblasts, the uptake of sarcosinamide at 22° C was determined at time points between 15 s and 30 min as shown in Fig. 2. The uptake of sarcosinamide was linear for up to 1 min. Intracellular levels of sarcosinamide were similar at 10 and 30 min, indicating that steady-state accumulation had been attained by 10 min. Since the uptake of sarcosinamide by the three cell lines was linear until 1 min, similar to previous determinations in SK-MG-1 and SKI-1 cells [10,

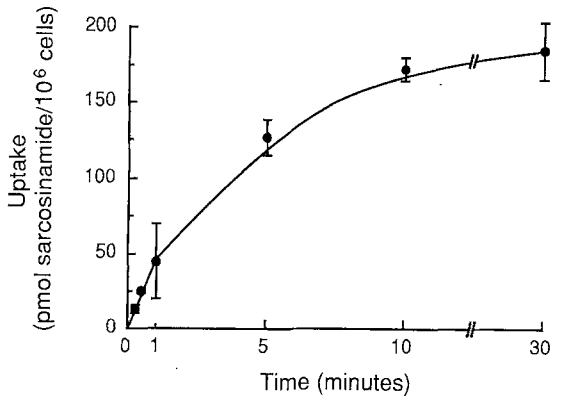

Fig. 2. Time course of the uptake of 0.05 mM *N*-[^{3}H]-sarcosinamide by monolayers of DHF fibroblasts at 22° C. The uptake is expressed as picomoles of tritiated sarcosinamide per 10^6 cells as described in Materials and methods. Points represent mean values for triplicate time points of a representative experiment, respectively; bars indicate the SE

11], all subsequent experiments were terminated at ≤30 s to approximate this initial rate of uptake conditions.

Presence of a sarcosinamide transporter

The presence of a sarcosinamide carrier in HT-29 colon cancer cells and in DHF fibroblasts was assessed by testing for potential inhibition with excess sarcosinamide (Table 1). A 200-fold excess of unlabeled sarcosinamide significantly decreased the uptake of tritiated sarcosinamide. A 72% reduction and a 25% decrease in the initial rate of uptake of sarcosinamide was observed in DHF fibroblasts and in HT-29 colon-cancer cells, respectively. In contrast, similar experiments using Calu-1 cells demonstrated no reduction in the uptake of tritiated sarcosinamide following the addition of 10 mM unlabeled sarcosinamide (data not shown).

Inhibition of sarcosinamide transport

Two physiological compounds were examined as potential inhibitors of sarcosinamide transport in HT-29 cells (Table 2). The catecholamine epinephrine, a potent inhibitor of sarcosinamide transport in SK-MG-1 glioma cells [11], and leucine, an amino acid transported by a neutral amino-acid transport system, were tested for sarcosinamide uptake suppression. In HT-29 cells, a 1,000-fold excess of epinephrine or leucine failed to decrease the uptake of sarcosinamide to any significant degree (Table 2). Similarly, neither compound significantly reduced the uptake of sarcosinamide in DHF fibroblasts.

Epinephrine transport

The next step was to determine the characteristics of epinephrine transport in the three cell lines. In HT-29 cells and in DHF fibroblasts, the uptake of 0.05 mM [^{3}H]-epinephrine at 30 s was not reduced by a 200-fold excess of epinephrine. The uptake of 0.05–10 mM [^{3}H]-epinephrine by Calu-1 cells at 30 s was investigated. A double reciprocal plot of the uptake of epinephrine at concentrations ranging from 0.05 to 10 mM shows a straight line going through the origin, consistent with passive diffusion. These results suggest the absence of an epinephrine carrier in all of these cell lines.

Table 1. Inhibition studies on the uptake of sarcosinamide in DHF fibroblasts and HT-29 colon cancer cells

Inhibitor	Uptake[a] (% of control)	*P*[b]
A. None (*N*-[^{3}H]-sarcosinamide)	100	
10 mM sarcosinamide	28 ± 10	<0.01
B. None (*N*-[^{3}H]-sarcosinamide)	100	
10 mM sarcosinamide	75 ± 2	<0.001

The 30-s uptake of 0.05 mM *N*-[^{3}H]-sarcosinamide at 22° C was examined in the presence or absence of a 200-fold excess of unlabeled sarcosinamide as described in Materials and methods. A, DHF fibroblasts; B, HT-29 colon cancer cells

[a] Mean values ± SE for 4 separate experiments

[b] Student's two-tailed *t*-test

Table 2. Effect of various inhibitors on the uptake of sarcosinamide by HT-29 colon cancer cells

Inhibitor	Concentration (mM)	Uptake[a] (% of control)	*P*[b]
None (*N*-[^{3}H]-sarcosinamide)		100	
Epinephrine	50	84 ± 7	NS
Leucine	50	93 ± 7	NS

The 30-s uptake of 0.05 mM *N*-[^{3}H]-sarcosinamide at 22° C was examined in the presence or absence of the indicated inhibitors as described in Materials and methods. NS, Not significant

[a] Mean values ± SE for 3 independent experiments

[b] Student's two-tailed *t*-test

Kinetics of sarcosinamide uptake

The 20-s uptake of sarcosinamide at concentrations ranging from 0.10 to 5.0 mM was tested in the HT-29 colon-cancer cell line. Figure 3 shows a double reciprocal plot for sarcosinamide uptake at 0.10–5.0 mM by HT-29 cells. The K_m value was found to be 2.76 ± 0.1 mM and the V_{max}, 2.03 ± 0.1 nmol/10^6 cells/min. The HT-29 cells showed a 10-fold reduction in the affinity of the sarcosinamide carrier and a 13-fold increase in its uptake capacity as compared with the results obtained in the SK-MG-1 cells.

The 30-s uptake of sarcosinamide at concentrations ranging from 0.05 to 20 mM was also examined in DHF fibroblasts. A Lineweaver-Burk plot for sarcosinamide uptake at 0.05–20 mM yielded a K_m value of 6.58 ± 3.90 mM and a V_{max} value of 12.08 ± 8.20 nmol/10^6 cells/min. The DHF fibroblasts showed more than a 20-fold decrease in the affinity of the sarcosinamide carrier and an approxi-

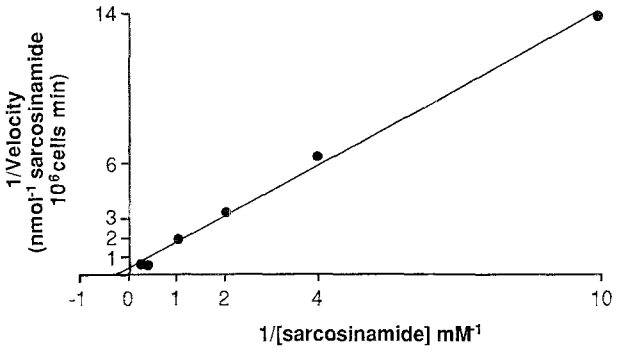

Fig. 3. Double reciprocal plot of the kinetics of sarcosinamide transport. The velocity of sarcosinamide uptake (nmol/10^6 cells/min) was determined after 20 s drug incubation at various concentrations of [^{3}H]-sarcosinamide (0.1–5.0 mM) in HT-29 colon cancer cells at 22° C as described in Materials and methods. Points represent mean values for 2 representative experiments. The results were analyzed by linear regression analysis ($y = 1.36\,x + 0.49$; $r = 0.998$)

mately 80-fold increase in its uptake capacity as compared with the results obtained in the SK-MG-1 cells.

In Calu-1 cells, the 30-s uptake of sarcosinamide was examined at concentrations ranging from 0.05 to 40 mM. The results show a linear relationship between the velocity of sarcosinamide uptake and the concentration of sarcosinamide, with no evidence of saturation of the transfer of sarcosinamide. This finding confirms the initial lack of evidence of inhibition of tritiated sarcosinamide uptake by excess unlabeled sarcosinamide in these cells.

Discussion

The objective of the present study was to determine the mode of transport of sarcosinamide in the three cell lines examined, i.e., whether sarcosinamide is taken up by a carrier-mediated process whose physiological function involves the transport of the catecholamine epinephrine, as has been shown in the SK-MG-1 human-glioma cell line [11].

The uptake of sarcosinamide by HT-29 cells and DHF fibroblasts was found to be competitively inhibited and saturable. These features are consistent with a carrier-mediated transport of sarcosinamide. Since both sarcosinamide and epinephrine share an *N*-methyl group and because epinephrine is the native substrate for the sarcosinamide carrier in SK-MG-1 cells [11], epinephrine was tested as a possible substrate for the sarcosinamide carrier. The neurotransmitter epinephrine does not appear to be the physiological compound for the carrier that accommodates sarcosinamide in these two cell lines. This observation is supported by the inability of epinephrine to inhibit sarcosinamide uptake. Moreover, epinephrine does not appear to enter HT-29 cells, Calu-1 cells, or DHF fibroblasts by a carrier-mediated mechanism. Due to the structural similarity of sarcosinamide to amino acids, leucine was considered as a possible physiological substrate for the carrier in HT-29 cells and DHF fibroblasts. The L-neutral amino-acid transport system, which accommodates leucine and amino acids with branched and aromatic side chains, does not appear to be the carrier that accommodates sarcosinamide in HT-29 cells and DHF fibroblasts. Therefore, in contrast to the situation in SK-MG-1 glioma cells, sarcosinamide is transported into HT-29 cells and DHF fibroblasts by a carrier system that does not involve the epinephrine carrier.

The kinetic parameters of the sarcosinamide carrier are very different from those observed in the SK-MG-1 cell line [11]. The carrier present in HT-29 cells and in DHF fibroblasts has a lower affinity and a higher capacity for sarcosinamide. Also, it is probable that sarcosinamide uptake occurs to some extent by passive diffusion in HT-29 cells, since a 200-fold excess of unlabeled sarcosinamide reduced the uptake of [^{3}H]-sarcosinamide by only 25%. In Calu-1 cells, the uptake of sarcosinamide occurs via passive diffusion.

The results of the present study provide evidence of a low-affinity, high-capacity transport system that accommodates sarcosinamide in two of the three cell lines tested. Sarcosinamide appears to be transported by a carrier that is different from the catecholamine carrier used in the SK-MG-1 glioma cell line. Thus, it may be concluded that there is neither a high-affinity sarcosinamide carrier nor an epinephrine carrier in the Calu-1 lung-cancer cell line, the HT-29 colon-cancer cell line, or in normal foreskin DHF fibroblasts. The increased sensitivity of glial tumors to SarCNU may be related to the presence of the catecholamine carrier that accommodates sarcosinamide and SarCNU, although we have thus far identified only one glial cell line, SK-MG-1, with a well-characterized epinephrine carrier [10, 11]. This carrier was not present in the colon-cancer cell line, the lung-cancer cell line, or, most importantly, the normal fibroblast cell strain.

Acknowledgements. This study was supported by National Institute of Neurological Disorders and Stroke grant RO1-NSC22230. The authors are grateful to Ms. A. McQuillan for experimental assistance and to Ms. S. Fraiberg for preparation of the manuscript.

References

1. Beigleiter A, Lam H-YP, Goldenberg GJ (1977) Mechanism of uptake of nitrosoureas by L5178Y lymphoblasts in vitro. Cancer Res 37: 1022–1027
2. Bodell WJ, Toduka K, Ludlum DB (1988) Differences in DNA alkylation products formed in sensitive and resistant human glioma cells treated with *N*-(2-chloroethyl)-*N*-nitrosourea. Cancer Res 48: 4489–4492
3. Carter SK (1973) An overview of the status of the nitrosoureas in other tumors. Cancer Chemother Rep 4: 35–46
4. Erickson LC, Laurent G, Sharkey NA, Kohn KW (1980) DNA cross-linking and monoadduct repair in nitrosourea-treated human tumour cells. Nature 288: 727–729
5. Gralla JD, Sasse-Dwight S, Poljak LC (1987) Formation of blocking lesions at identical DNA sequences by the nitrosourea and platinum classes of anticancer drugs. Cancer Res 47: 5092–5096
6. Panasci L, Dufour M, Chevalier L, Isabel C, Lazarus P, McQuillan A, Arbit E, Brem S, Feindel W (1985) Utilization of the HTSCA and CFU-C assay to identify two new chloroethylnitrosourea congeners of amino acid amides with increased in vitro activity against human gliomas as compared to BCNU. Cancer Chemother Pharmacol 14: 156–159

7. Robins P, Harris AL, Goldsmith L, Lindahl T (1983) Cross-linking of DNA induced by chloroethylnitrosourea is prevented by O^6-methylguanine-DNA methyltransferase. Nucleic Acid Res 11: 7743–7758
8. Ronquist G, Agren G, Ponten J, Westermark B (1976) α-Amino-isobutyric acid transport into human glia and glioma cells in culture. J Cell Physiol 89: 433–440
9. Skalski V, Rivas J, Panasci L, McQuillan A, Feindel W (1988) The cytotoxicity of sarcosinamide chloroethylnitrosourea (SarCNU) and BCNU in primary gliomas and glioma cell lines: analysis of data in reference to theoretical peak plasma concentrations in man. Cancer Chemother Pharmacol 22: 137–140
10. Skalski V, Yarosh DB, Batist G, Gros P, Feindel W, Kopriva D, Panasci LC (1990) Mechanisms of resistance to 2-chloroethyl-3-sarcosinamide-1-nitrosourea (SarCNU) in sensitive and resistant human glioma cells. Mol Pharmacol 38: 299–305
11. Skalski V, Feindel W, Panasci LC (1990) Transport of amino acid amide sarcosinamide and sarcosinamide chloroethylnitrosourea in human glioma SK-MG-1 cells. Cancer Res 50: 3062–3066
12. Smith DG, Brent TP (1989) Response of cultured human cell lines from rhabdomyosarcoma xenografts to treatment with chloroethylnitrosoureas. Cancer Res 49: 883–886
13. Smith MT, Evans CG, Doane-Setzer P, Castro VM, Tahir MK, Mannervik B (1989) Denitrosation of 1,3-bis(2-chloroethyl)-1-nitrosourea by class Mu glutathione transferases and its role in cellular resistance in rat brain tumor cells. Cancer Res 49: 2621–2625
14. Suami T, Kato T, Hisamatsu T (1982) 2-Chloroethylnitrosourea congeners of amino acid amides. J Med Chem 25: 829–832
15. Walker MD (1973) Nitrosoureas in central nervous system tumours. Cancer Chemother Rep 4: 21–26