2,2′-Dithiobis(N-ethyl-spermine-5-carboxamide) Is a High Affinity, Membrane-impermeant Antagonist of the Mammalian Polyamine Transport System*

(Received for publication, June 21, 1996, and in revised form, August 13, 1996)

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We have synthesized 2,2′-dithiobis(N-ethyl-spermine-5-carboxamide) (DESC), its thiol monomer (MESC), and the mixed MESC-cysteamine disulfide (DEASC) as potential inhibitors of polyamine transport in mammalian cells. DESC was the most potent antagonist of spermine transport in ZR-75-1 human breast cancer cells, with Ki values of 5.0 ± 0.7, 80 ± 31, and 16 ± 3 µM for DESC, MESC, and DEASC, respectively. DESC also strongly blocked putrescine and spermidine uptake in ZR-75-1 cells (Ki = 1.6 ± 0.5 and 2.7 ± 1.1 µM, respectively). While DESC and MESC were purely competitive inhibitors of putrescine transport, DEASC was a mixed competitive/noncompetitive antagonist. Remarkably, DESC was virtually impermeant in ZR-75-1 cells despite its low Ki toward polyamine transport. The marked difference in affinity between DESC and MESC was essentially due to the tail-to-tail juxtaposition of two spermine-like structures, suggesting that dimeric ligands of the polyamine transporter might simultaneously interact with more than one binding site. While DESC strongly decreased the initial rate of [3H]spermidine transport, even a 40-fold molar excess of antagonist could not completely abolish intracellular spermidine accumulation. Moreover, as little as 0.3 µM spermidine fully restored growth in ZR-75-1 cells treated with an inhibitor of polyamine biosynthesis in the presence of 50 µM DESC, thus emphasizing the importance of uptake of trace amounts of exogenous polyamines. Thus, reducing the exogenous supply of polyamines with a potent competitive inhibitor may be kinetically inadequate to block replenishment of the polyamine pool in polyamine-depleted tumor cells that display high transport capacity. These results demonstrate that polyamine analogues cross-linked into a dimeric structure such as DESC interact with high affinity with the mammalian polyamine carrier without being used as substrates. These novel properties provide a framework for the design of specific irreversible inhibitors of the polyamine transporter, which should present advantages over competitive antagonists for an efficient blockade of polyamine transport in tumor cells.

Natural polyamines (putrescine, spermidine, and spermine) play essential roles in the control of macromolecular synthesis and cell growth in eukaryotes (1–3). Although most tissues can synthesize polyamines, they also possess a specific plasma membrane transport system, which allows utilization of plasma sources of polyamines and the salvaging of polyamines spontaneously excreted by mammalian cells (4–6). Although eukaryotic polyamine transport has been the focus of numerous studies, no molecular information on the carrier molecules is yet available.

Several specific inhibitors of polyamine biosynthesis have been designed, such as the ornithine decarboxylase suicide substrate, α-difluoromethylornithine (DFMO),1 which deplete polyamines with subsequent growth arrest in virtually all known mammalian cell types in culture (1, 3, 7). Based on these premises, DFMO has been extensively assessed for the treatment of proliferative diseases, including several tumor types, in experimental models and in clinical trials (1, 3), but its in vivo antitumor efficacy was found to be limited. The failure of DFMO to halt tumor growth in animal models has been correlated with the elevated polyamine transport activity found in transformed cells and its up-regulation as a result of polyamine depletion (4, 6). Thus, decontamination of the gastrointestinal tract, which is the main vector of circulating polyamines through bacterial microflora activity, along with a polyamine-free diet (8, 9), markedly potentiate the in vivo efficacy of DFMO against tumor progression (10, 11). Moreover, mutant leukemia cells deficient in polyamine transport are much more susceptible than parental cells to growth inhibition by DFMO in vivo (10, 12). Thus, polyamine transport has major implications for antitumor therapies based on polyamine depletion.

An obvious strategy to block accumulation of exogenous polyamines by tumor cells might be the use of a specific transport antagonist. Ideally, candidate molecules should have high affinity for binding to the polyamine carrier but should not be internalized by the polyamine transport system, or if they are substrates, they should not mimic the biological activities of natural polyamines. In the course of studies on the structure of the polyamine transporter, we have synthesized N-(2-mercapto-

* This project was supported by National Sciences and Engineering Council of Canada Strategic Grant 0149324. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: DFMO, α-difluoromethylornithine; MESC, N-(2-mercaptoethyl)-spermine 5-carboxamide; DESC, 2,2′-dithiobis(N-ethyl-spermine-5-carboxamide); FABMS, fast atomic bombardment spectrometry; LSIMS, liquid secondary ion mass spectrometry; DEASC, N-22,2′-dithio(ethy1,1′-aminoethyl)-spermine 5-carboxamide; LY, lucifer yellow; ASIB, 1-(p-azidosalicylamido)-4-(iodoacetamido)butane; Boc, tert-butyl carbonyl; PBS, fetal bovine serum; SAI0, serum amine oxidase; HPLC, high performance liquid chromatography; DTT, dithiothreitol; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; CHX, cycloheximide.
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EXPERIMENTAL PROCEDURES

Materials and Reagents—Ornithine dihydrochloride, dimethylformamide, and other reagents for organic synthesis were purchased from Aldrich and Sigma. Reversed phase silica gel liquid chromatography reagents and tissue culture reagents were from Sigma. Reagents for high performance liquid chromatography (HPLC) were obtained from Fisher Scientific (Montreal, Quebec, Canada) or Aldrich. Other biochemical reagents and tissue culture reagents were from Sigma.

Synthesis of 5-Carboxyspermine—Unless otherwise indicated, reactions were performed at room temperature. 5-Carboxyspermine (I, Fig. 1) was synthesized using the scheme proposed by Behr et al. (13). Briefly, to a stirred solution of 10.0 g (59.3 mmol) of ornithine hydrochloride dissolved in 250 ml of MeOH were added 18.0 g (197 mmol) of tetramethylammonium hydroxide. After dissolution of ornithine salt, MeOH was removed, the mixture was then desalted in 350 ml of thin-layer chromatography (TLC), and the residual ammonium salt was filtered, yielding ornithine as its free base. Following the addition of acrylonitrile (2.2 eq, 130.9 mmol), the mixture was stirred for 16 h in the dark to give 10.5 g of crude N,N'-diethyldithiocarbamic acid, which was subsequently used without further purification. White solid; IR (film) ν cm⁻¹ 3007 (OH), 2969 (CH), 1706 (C=O).

To obtain 5-carboxyspermine (I), COH (2.7 g, 48.0 mmol) was dissolved with vigorous stirring in 8 ml of 95% (v/v) EtOH and 10.5 g (44.1 mmol) of N,N'-diethyldithiocarbamic acid were then added. The resulting mixture was placed under H₂ at 40 p.s.i. in a Burgess-Parr hydrogenator, and stirring was continued for 2.09 h (24.4 mmol) of Raney nickel as catalyst (13, 14). After 22 h, Raney nickel was removed by filtration, and the solvent was evaporated in vacuo, yielding 16.1 g of the crude 5-carboxyspermine potassium salt. Yellow oil; IR (film) ν cm⁻¹ 3363 (OH, acid), 2937 (NH₂), no cyanide band; 1H NMR δ (CDCl₃) 1.53 (m, 2H, CH₂COOH), 1.65 (m, 6H, CH₃CH₂CH₂), 2.51 (m, 4H, CH₂NH₂), 2.65 (m, 6H, CH₂NH), 3.09 (t, 2H, CH₂COOH).

SYNTHESIS OF DESC AND N-[2,2'-Dithio(ethyl,1'-aminomethyl)-5-carboxyspermine (DEASC)]—The compound (IV, Fig. 1) was synthesized using the scheme proposed by Ponnusamy et al. (15). To 16.0 g of crude 5-carboxyspermine potassium salt dissolved in 1.5 liters of MeOH were added 9.64 ml of 10% (v/v) triethylamine and 54.3 g (4.4 eq, 286 mmol) of di-t-butyl dicarbonate. After stirring for 24 h, the solvent was evaporated, 100–150 ml of H₂O were added, and the resulting mixture was chilled at 0 °C. After adjustment of pH to 7.2 with NaOH in the presence of Boc-protected spermine, the mixture was extracted with ethyl acetate, dried over anhydrous MgSO₄, and purified by C₁₈ reversed phase silica gel chromatography, yielding 3.3 g of pure teta-Boc-5-carboxyspermine (II, Fig. 1). Yield of II from l-ornithine was 11%. Light yellow solid; IR (film) ν cm⁻¹ 3558 (OH, acid), 1652 (C=O, amide). 1H NMR δ (CDCl₃) 1.32 (2H, 36s, OC(CH₃)₃), 1.90–1.40 (m, 40H, CH₂CH₂, CH₂CO, CH₂NCOOCH₂CH₃), 2.50–2.67 (4C, CH₂NHCOOCH₂CH₃, CH₂COOCH₂CH₃), 2.83 (12C, OC(CH₃)₃), 37.30 (2C, CH₂NCOOCH₂CH₃), 43.73–46.55 (3C, CH₂NCOOCH₂CH₃), 59.31 and 60.22 (1C, CH₂COO), 78.92, 79.67, and 80.72 (4C, OC(CH₃)₃), 154.46 and 156.02 (4C, COO(CH₂CH₃)), 174.81 (1C, COOCH₂). M (for C₆H₁₀O₄N₁₁) = 646.41; m/z (LSIMS) = 647.42 (M + Δ⁻²). The crude product was then purified by reversed phase HPLC chromatography, yielding 0.682 g of 2,2'-dithiobis[N-(N',N',N'')-tetra-Boc-5-carboxyspermine] (III, Fig. 1) and 0.124 g of N-[2,2'-dithio(ethyl,1'-aminomethyl)-N',N',N''-triacetic acid]—tetra-Boc-spermine-5-carboxamide (IV, Fig. 1). Yield was 27.2 and 8.9% for III and IV, respectively.

III, yellow oil; IR (film) ν cm⁻¹ 1693 (C=O, amide). 1H NMR δ (CDCl₃) 1.38 (3H, CH₃(C), 1.59 (m, 6H, CH₂CH₂, CH₂CO, CH₂NCOOCH₂CH₃), 2.53 (t, 1H, CONHCH₂CH₂CH₂), 2.78 (t, 2H, CH₂S), 3.11 (m, 10H, CH₂NH), 3.51 (2H, CH₂CH₂H), 3.78 (m, 2H, CONHCH₂CH₂S), 4.11 (t, 1H, CHCONH); 13C NMR δ (CDCl₃) 25.62–29.76 (4C, CH₂NHCOOCH₂CH₃, CH₂NCOOCH₂CH₃ and CH₂COOCH₂CH₃), 39.19 (4C, CH₂NH₂), 40.75 (2C, CH₂NHCO), 46.32 (2C, CH₂S), 62.32 and 62.78 (2C, CH₃CO), 78.96–79.49 (8C, OC(CH₃)₃), 156.00–156.20 (2C, COO(CH₂CH₃), 174.81 (2C, CH₂COO), M (for C₆H₁₀O₄N₁₁) = 609.4 (M + Δ⁻²). Compound III (215 mg in MeOH) was then deprotected by the addition of 1 ml of 3 M HCl, which brought the pH from 6.0 to ~0.5. After stirring vigorously for 15 h, the solution was dried out in vacuo, and the resulting compound was purified by cation exchange chromatography with a Dowex-50WX4 column (dry mesh: 100–200; Sigma) pre-equilibrated with H₂O and successively washed with H₂O, 1 x HCl, 2 x HCl, 4 x HCl, and 6 x HCl. Ninyhdrin-positive fractions eluted with 6 x HCl were pooled and evaporated in vacuo, yielding 96 mg of pure DESC (V, Fig. 1). Yield was 97%. White solid; m.p. 75–78 °C; b.p. 118 °C. 1H NMR δ (CDCl₃) 1.62 (m, 2H, CH₂CONH), 1.97–1.80 (8H, CH₂CH₂), 2.74 (2H, CH₂S), 2.92 (m, 10H, CH₂NH), 3.46 (dt, 2H, CH₂CH₂S), 3.54 (t, 2H, CONH), 3.94, 29.34, 26.33, 28.58, and 29.58 (8C, CH₂CH₂NHCH₂CH₂NHCH₂CH₂NHCH₂CH₂NCOOCH₂CH₃, and CH₂COOCH₂CH₃), 38.69 (2C, CH₂COO), 39.19 (4C, CH₂NH), 40.75 (2C, CH₂NHCOOCH₂CH₃), 46.39, 47.23, and 49.44 (8C, CH₂NH), 62.83 (2C, CH₂COO), 170.28 (2C, CO₂). M (for C₆H₁₂O₄N₁₁S₂) = 608.96; m/z (FABMS) = 609.4 (M⁺). Overall yield of V from l-ornithine was 3.1%.

Compound IV was similarly deprotected to yield DESAC (VI, Fig. 1). Yellow solid; m.p. 50–54 °C; b.p. 109 °C. 1H NMR δ (CDCl₃) 1.89 (m, 2H, CH₂CONH), 2.10–2.29 (m, 6H, CH₂CH₂CH₂), 3.04 (t, 2H, CONHCH₂CH₂), 3.19 (t, 2H, SCCH₂CH₂NH), 3.25 (m, 10H, CH₂NH), 3.51 (t, 2H, SCCH₂CH₂NH), 3.78 (m, 2H, CONHCH₂CH₂), 4.11 (t, 1H, CHCONH); 13C NMR δ (D₂O) 23.96, 26.33, and 29.58 (4C, CH₂CH₂NHCH₂CH₂NHCH₂CH₂NCOOCH₂CH₃, and CH₂COOCH₂CH₃), 38.69 (2C, CH₂COO), 39.19 (4C, CH₂NH), 40.75 (2C, CH₂NHCOOCH₂CH₃), 46.39, 47.23, and 49.44 (8C, CH₂NH), 62.83 (2C, CH₂COO), 170.28 (2C, CO₂). M (for C₆H₁₀O₄N₁₁S₂) = 608.96; m/z (FABMS) = 609.4 (M⁺). Overall yield of VI from l-ornithine was 0.98%.

Synthesis of MESC—DESC was dissolved in sodium phosphate buffer (50 mM, pH 8.0) containing 250 mM diethiothreitol (DTT) and...
incubated for 30 min at 37 °C. The mixture was then loaded on a Dowex 50W-X4 cation exchange column equilibrated with H2O. After washing with 5 column volumes each of 1 N HCl and 2 N HCl, the free thiol was eluted with 10 volumes of 4 N HCl. Amine-containing fractions were identified by mixing 5-μl aliquots with 200 μl of an o-phthalaldehyde dehyde solution (3.7 mM o-phthalaldehyde; 0.4 mM boric acid, pH 10.4; 1% (v/v) MeOH; 0.45% (v/v) 2-mercaptoethanol; 0.03% (v/v) Brij 35) and heating for 20 min at 37 °C and then cooled. The amount of MESC was determined by measuring the absorbance of the o-phthalaldehyde derivative at 356 nm, using a standard. Theyield of MESC using this procedure was virtually 100%, based on the number of thiol equivalents determined with 5,5′-dithio-bis-(2-nitrobenzoic acid) (17) using either cysteamine or DTT as a standard. The yield of MESC from this procedure was virtually 100%, based on the number of thiol equivalents determined with 5,5′-dithio-bis-(2-nitrobenzoic acid) and the expected number of thiol equivalents per mass of DES. Finally, MESC purity was confirmed by ion pair reverse phase HPLC using postcolumn derivatization with o-phthalaldehyde (18). 1H NMR δ (DCCl3) 1.91 (m, 2H, CH2CHONH), 2.08–2.24 (m, 6H, CH2CH2CH2), 2.82 (t, 2H, CONHC=CH2SH), 3.22 (m, 10H, CH2NH), 3.56 (m, 2H, CONHC=CH2SH), 4.11 (t, 1H, CH=ONH); 13C NMR δ (D2O) 23.87, 26.28, and 26.49 (4C, CH2CH2NH, CH2CH2CH2, and CH2CONH), 25.67 (1C, CH3H), 39.09 (1C, CH=CH2), 40.90 (2C, CH2NH), 46.30, 47.13, and 49.35 (3C, CH2CONH, HNH, and CH2CONH), 62.79 (1C, CH2CHO) and 170.24 (1C, COO−).

Synthesis of Thioether Adducts of MESC with Iodoacetamides—To 1 ml of an extemporaneously prepared, DTT-free solution of MESC (20 μg/ml in H2O) were added 50 μl of 50 mM Tris-HCl (pH 7.0) and 105 μl of a 40 mM solution of iodoacetamide, L-tyrosine iodoacetamide, or ASIB in a light-protected microcentrifuge tube, and the mixture was incubated for 2 h at 37 °C. After the incubation completion as determined by measuring the amount of thiol remaining at the end of the incubation with 5,5′-dithio-bis-(2-nitrobenzoic acid) as described above. Excess iodoacetamide was then inactivated by adding DTT to a final concentration of 40 mM and incubating the solution for 2 h at 37 °C. The resulting solutions of MESC adducts were used without further purification for [3H]spermidine uptake assay. In some experiments, ZR-75-1 cells were also grown in Richter's minimal essential medium supplemented with 5% (v/v) dextran-coated charcoal-treated FBS (SD medium), as indicated otherwise. Inhibitors were added to culture medium for 11 days in medium supplemented with antagonist, polyamines, and/or inhibitors on cell growth was measured by incubating ZR-75-1 cells for 250 μg/ml bovine insulin, and 105 cells/dish in MEZR medium and grown for 5 days.

Cell Culture—Both ZR-75-1 human breast cancer cells and Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (Rockville, MD). ZR-75-1 cells were maintained in phenol red-free RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 15 mM Hepes, 10 mM β-estradiol, and antibiotics (MEZR medium) (19). CHO-K1 cells were routinely grown in α-minimal essential medium supplemented with 10% (v/v) FBS, 2 mM l-glutamine, and 15 mM Hepes, and antibiotics, 1 mM 17β-estradiol, and polyamines. In some experiments from triplicate cell cultures.

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incubation was then determined by incubating ZR-75-1 cells with transport inhibitors dissolved in MEZR medium containing 5 μM [3H]spermidine in the presence or absence of 200 μM CHX. For determination of spermidine uptake activity in CHO-K1 cells, 80% confluent cell monolayers were rinsed twice with PBS and incubated for 20 min at 37 °C in 400 μl of buffer A (20 mM Tris-HCl, pH 7.4, 0.42% NaCl, 0.1% bovine serum albumin (Fraction V), 0.02% (v/v) Triton X-100, 10 mM NaCl) containing 5 μM [3H]spermidine (20 Ci/mol). Cell cultures were then washed twice with 1 ml of PBS containing 5.7 mM sodium selenite. Cells were then lysed with 200 μl of 1 N NaOH and incubated for 30 min at 60 °C. After neutralization with 200 μl of 1 N HCl, radioactivity was determined from 250 μl of the cell lysate by scintillation counting. Uptake activity was expressed per amount of total cellular protein. Kinetic parameters of polyamine transport were determined by Lineweaver-Burke analysis of uptake activity in the presence of 0.3 μM [3H]putrescine or 0.1 μM [3H]spermine plus increasing concentrations of nonradioactive substrate. For competitive inhibitors, Ki values were also estimated by iterative curve fitting for sigmoidal equations describing transport rates in the presence of increasing concentrations of antagonist. For mixed competitive/noncompetitive inhibition, two methods were used to calculate kinetic constants. First, the equation,
chromatography (data not shown); thus, most DESC preparations contained a small amount (1–2%) of DEASC after reversed phase liquid chromatography on C18 silica gel. DESC and DEASC were stable for months at 220°C in aqueous solutions buffered at pH 7.0, whereas MESC solutions were supplemented with DTT to prevent oxidation.

Affinity of DESC, DEASC, and MESC for the Mammalian Diamine and Polyamine Transport Systems—In order to evaluate the suitability of the spermine conjugates as prospective affinity ligands, their relative ability to inhibit putrescine and polyamine uptake was evaluated. DESC was the most potent antagonist of [14C]spermine transport in ZR-75-1 cells, with a Ki value about 5- and 16-fold lower than that of DEASC and MESC, respectively (Fig. 2). Moreover, the ability of spermine to compete against [3H]putrescine and [3H]spermidine uptake was in fact only about 7-fold higher than for DESC (Fig. 3). DESC (Fig. 4A) and MESC (data not shown) were pure competitive inhibitors of [3H]putrescine uptake at concentrations up to 100 and 200 μM, respectively. On the other hand, inhibition of putrescine transport by DEASC belonged to a mixed competitive/noncompetitive type (Fig. 4B). Table I summarizes the Ki values determined for DESC, MESC, and DEASC toward putrescine, spermidine and/or spermine uptake, in relation with the mutual transport interactions between the latter substrates. Notably, Ki values of the three spermine conjugates with respect to putrescine uptake were 3–5-fold lower than for spermine uptake, unlike spermidine and spermine, which both inhibited the uptake of either substrate with similar potency and with a Ki roughly equal to their Km as substrate. In CHO cells, DESC and MESC competitively inhibited spermidine uptake, with Ki values of 0.92 ± 0.15 and 33.6 ± 7.2 μM, respectively (data not shown; cf. Fig. 5).

Effect of Side Chain Length and Substituents on Spermidine Transport Inhibition by MESC Derivatives—The observation that MESC was a less potent inhibitor of di- and polyamine transport than DESC or DEASC suggested that the nature of the side chain influences the interaction of these compounds with the carrier. MESC was thus derivatized with substituting groups of various sizes and charges through thioether linkage with three different iodoacetamides, namely LY iodoacetamide, ASIB, and iodoacetamide itself, and the ability of the resulting conjugates (MESC-LY, MESC-ASIB, and MESC-acetamide, respectively) to inhibit spermidine uptake was then evaluated. These studies were conducted in CHO-K1 cells, which we have used to identify the polyamine carrier protein(s) by labeling with 125I-labeled MESC-ASIB and modification reagents such as carbodiimides (26). As shown in Fig. 5, derivatization of the thiol group of MESC did not significantly (p > 0.10) increase the Ki toward spermidine uptake for the three conjugates studied. In the case of MESC-ASIB, Ki values might have been underestimated by partial inactivation of the polyamine carrier at the assay temperature, although the uptake reaction was conducted under subdued lighting. Thus, specific recognition of the spermine head of MESC can accommodate considerable variation in length, size, polarity, or charge for the side chain without detrimental effect on its affinity for the polyamine carrier.

Lack of Permeation of DESC and MESC through the Polyamine Transport System—A number of polyamine analogues are effective competitors of polyamine uptake while being themselves substrates for transport (4, 5, 27–30). These analogues share many structural features with natural polyamines and can be used as substitutes, or they have cytotoxic effects in mammalian cells alone or in combination with DFMO (3, 5,

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**Fig. 1.** Scheme for the synthesis of DESC, DEASC, and MESC. Details of the synthesis are as given under "Experimental Procedures." a, triethylamine; b, di-tert-butyl dicarbonate; c, cyanuric chloride; d, cystamine dihydrochloride; e, 3 N HCl; f, dithiothreitol; g, 50 mM sodium phosphate in aqueous solution (pH 8.0).

**Fig. 2.** Inhibition of [14C]spermine transport by MESC, DESC, and DEASC in human ZR-75-1 breast cancer cells. The rate of spermine uptake was measured in ZR-75-1 cells grown as monolayers in 24-well culture plates in the presence of the indicated concentrations of DESC (E), MESC (●), and DEASC (M), using 1 μM [14C]spermine as substrate. Data are the mean ± S.D. of triplicate determinations.

**Fig. 3.** Inhibition of [3H]putrescine and [3H]spermidine transport by MESC, DESC, and DEASC in ZR-75-1 breast cancer cells. The rate of putrescine uptake was measured in ZR-75-1 cells grown as monolayers in 24-well culture plates in the presence of the indicated concentrations of DESC (E), MESC (●), and DEASC (M), using 1 μM [3H]putrescine as substrate. Data are the mean ± S.D. of triplicate determinations.
27–29, 31–34). On the other hand, the availability of high affinity and impermeant antagonists of polyamine transport would allow us to evaluate the antitumor efficacy of polyamine depletion strategies in vivo with minimal systemic cytotoxic effects.

The ability of ZR-75-1 cells to accumulate DESC and MESC was thus determined. Since DESC was eluted as a late, broad peak in the HPLC system used, DTT was added to cell extracts to reduce DESC to MESC, which eluted earlier, and to decrease the detection threshold. As shown in Table II, only trace amounts of DESC could be recovered in ZR-75-1 cells after a 6-h incubation with 200 μM DESC. These levels represent only about 1.5% of the accumulation measured for spermine (B) or spermidine (A) as substrate. Data are the mean ± S.D. of triplicate determinations from a representative experiment.

![Fig. 3. Inhibition of [3H]putrescine and [3H]spermidine uptake by spermine and DESC in ZR-75-1 cells. The rate of spermidine uptake was measured in ZR-75-1 cells grown as monolayers in 24-well culture plates in the presence of the indicated concentrations of spermine (○) and DESC (●), using 3 μM [3H]putrescine (A) or 1 μM [3H]spermidine (B) as substrate. Data are the mean ± S.D. of triplicate determinations from a representative experiment.](Image 315x320 to 547x498)

![Fig. 4. Lineweaver-Burk analysis of putrescine transport inhibition by DESC and DEASC in ZR-75-1 cells. The rate of [3H]putrescine uptake was determined in ZR-75-1 cell cultures with increasing concentrations of substrate in the presence of 0 (○), 3 (●), 10 (▲), 30 (■), or 100 μM DESC (▲) or in the presence of 0 (○), 20 (●), 50 (▲), or 200 μM DEASC (■).](Image 54x623 to 292x729)

![FIG. 5. Structure of MESC thioether derivatives and their Ki values with respect to spermidine uptake in CHO-K1 cells. The various conjugates were prepared from MESC as described under “Experimental Procedures”; the structures and names of the substituents are given in the first two columns from the left. R corresponds to the group attached to sulfur in MESC (cf. structure X, Fig. 1). The rate of spermidine uptake was determined in CHO-K1 cells in the presence of increasing concentrations of the various MESC derivatives, using 1 μM [3H]spermidine as substrate. Ki values are given as the mean ± S.D. of triplicate determinations from two or three experiments.](Image 66x431 to 280x540)

### Table I

| Compound      | Putrescine | Spermidine | Spermine |
|---------------|------------|------------|----------|
| Ki (μM)       |            |            |          |
| Putrescine    | 3.7 ± 0.4a | 125 ± 29a  | 0.23 ± 0.13b |
| Spermidine    | 0.23 ± 0.05a | 0.49 ± 0.15a | 0.37 ± 0.09b |
| Spermine      | 0.33 ± 0.02b | ND         | 0.20 ± 0.06b |
| DESC          | 1.6 ± 0.5b  | 2.7 ± 1.1b  | 5.0 ± 0.7b |
| MESC          | 22 ± 3b     | ND         | 80 ± 31b  |
| DEASC         | 5.3 ± 0.6 (Ki) | ND      | 16 ± 3”   |

a From Ref. 23.

b This work; mean ± S.D. of triplicate determinations from two to four different experiments.

| R              | Name             | Ki (μM) |
|----------------|------------------|---------|
| H              | MESC             | 33.6 ± 7.2 |
| CH₃-S-NH₂      | MESC-acetamide   | 48.9 ± 9.1 |
| CH₃-S-NR₂      | MESC-LY          | 44.1 ± 8.8 |
| CH₂-S-NR₂      | MESC-ASIB⁺       | 18.3 ± 8.2 |

a Value for Ki is likely underestimated due to partial irreversible inhibition of the polyamine transporter (cf. "Results").
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TABLE II
Intracellular accumulation of DESC and MESC in ZR-75–1 cells

| Addition     | Time | Polyamine intracellular contents | nmol/mg protein |
|--------------|------|----------------------------------|----------------|
|              |      | Spermidine | Spermine | DESC | MESC |
| Control      | 1    | 0.69 ± 0.08 | 8.22 ± 0.48 | 9.16 ± 0.13 | 9.47 ± 0.13 |
|              | 6    | 0.80 ± 0.07 | 8.27 ± 0.48 | <0.01 | <0.01 |
| 50 μM DESC   | 1    | 0.80 ± 0.14 | 8.60 ± 0.29 | <0.01 | <0.01 |
|              | 6    | 0.73 ± 0.11 | 8.77 ± 0.79 | <0.01 | <0.01 |
| 200 μM DESC  | 1    | 0.76 ± 0.11 | 8.66 ± 0.26 | 0.12 ± 0.01 | <0.01 |
|              | 6    | 0.68 ± 0.11 | 8.57 ± 0.31 | <0.01 | <0.01 |
| 200 μM DESC and CHX | 1 | 0.70 ± 0.03 | 9.55 ± 0.13 | 0.30 ± 0.01 | <0.01 |
|              | 6    | 0.80 ± 0.03 | 8.93 ± 0.35 | <0.01 | <0.01 |
| 50 μM MESC   | 1    | 0.95 ± 0.10 | 7.77 ± 0.06 | <0.01 | <0.01 |
|              | 6    | 0.75 ± 0.11 | 8.13 ± 0.17 | <0.01 | <0.01 |
| 200 μM MESC  | 1    | 1.15 ± 0.07 | 8.93 ± 0.53 | <0.01 | 0.20 ± 0.005 |
|              | 6    | 0.81 ± 0.15 | 8.32 ± 0.43 | <0.01 | 0.13 ± 0.06 |

*Significantly different (p < 0.05) from control value at time = 1 h.

FIG. 6. Effect of DESC and MESC on the intracellular accumulation of [3H]spermidine in ZR-75-1 cells. A, at time zero, 5 μM [3H]spermidine was added to ZR-75-1 cell cultures grown in 24-well plates (1 ml/well), in the presence of 50 or 200 μM DESC or MESC prior to determination of polyamine contents. CHX was added at 200 μM where indicated. Other details are provided under “Experimental Procedures.” Values are the mean ± S.D. of triplicate determinations from two independent experiments.

FIG. 7. Effect of spermidine, MESC, DESC, and DEASC on ZR-75-1 cell proliferation. Cells were incubated for 11 days in MEZR medium with the indicated concentration of spermidine, DESC, MESC, or DEASC in the presence (shaded bars) or absence (plain bars) of 1 mM aminoguanidine, and DNA content per culture was then determined. Data represent the mean ± S.D. of triplicate determinations.

copper amine oxidase.

Effect of DESC on Prevention of DFMO-induced Growth Inhibition by Exogenous Spermidine—Although DESC is indeed a potent antagonist of polyamine accumulation, the slow residual uptake that occurred even at a 40-fold molar excess of inhibitor might be sufficient to counteract polyamine depletion by inhibitors of polyamine biosynthesis. This possibility was assessed by comparing the ability of DESC to prevent the counteraction of DFMO-induced growth inhibition by exogenous spermidine. At concentrations >0.3 μM, spermidine inhibited ZR-75-1 cell proliferation by up to 20% (Fig. 8). This effect could be due to an incomplete inhibition of SAO by aminoguanidine (36), since it was not observed in media supplemented with equine serum, which does not contain SAO activity (37), instead of FBS (data not shown). The approximately 50% growth inhibition induced by 1 mM DFMO was completely reversed by as little as 0.3 μM spermidine, whereas 0.1 μM spermidine already restored growth of DFMO-treated cells to 78% of control value. However, the addition of 50 μM DESC was unable to prevent the reversal of DFMO-induced growth inhibition by spermidine, even at a DESC:spermidine ratio of 500. Essentially similar results were obtained using horse serum instead of FBS, or replacing RPMI 1640 medium, which contains 3.2 μM glutathione that might undergo thiol/disulfide exchange with DESC, with thiol-free Richter’s improved minimum essential medium (data not shown).

DISCUSSION

We have shown that DESC, a novel type of spermine analogue, is endowed with high affinity for the polyamine trans-
port system while being highly resistant to cellular uptake. The combination of these two attributes confers unique characteristics on DESC as a pure competitive antagonist of polyamine uptake. Only few attempts have been made previously to design specific inhibitors of polyamine transport. Based on the finding that parquat (4,4'-bipyridine) is a substrate of the putrescine transport system (38, 39), Minchin et al. (40) have synthesized a series of polypyridinium salts, including compounds with a low Kᵢ against putrescine uptake and low acute toxicity for mammalian cells. However, it is unclear whether such compounds can efficiently inhibit polyamine transport or are accumulated intracellularly. More recently, a high molecular mass (~25-kDa) spermine polymer has been described as a competitive inhibitor of polyamine transport with a Kᵢ in the 10⁻⁶ M range, but its usefulness at specifically blocking polyamine accumulation is uncertain because of its marked cytotoxicity (41).

As compared with spermine, the higher Kᵢ of MESC against putrescine, spermidine, and spermine uptake could be due to the presence of an amide linkage, which decreases the basicity of the neighboring secondary amino group of the spermine head (pKᵢ ~5.5 in comparison with 8.9–9.8 for spermine) (2, 42), and/or may cause steric hindrance for its interaction with the polyamine binding site (27, 28). Nevertheless, despite the unfavorable structural features of MESC as a ligand, its dimerization into DESC increased by ~20-fold the affinity of the resulting structure for the polyamine transporter. Although there is no precedent for dimeric polyamine structures like DESC, its overall design is reminiscent of that of 2-N-4-(1-azido-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-proplylamine, an impermeant ligand that binds to the exofacial domain of facilitative glucose transporters and bears two symmetrical sugar moieties linked tail to tail (43). It is noteworthy that at least one mammalian glucose transporter, namely GLUT-1, exists as a tetrameric complex in its native form (44, 45). It is therefore conceivable that the stronger affinity of DESC relative to MESC could reflect a dyad symmetry in the organization of the transporter complex. Alternatively, dimerization of MESC into DESC could impose conformational constraints (e.g., due to electrostatic repulsion) that would favor recognition of the polyamine binding site of the carrier by each of the symmetrical spermine moieties.

MESC thioethers as diverse in size as MESC-LY, MESC-ASIB, or MESC-acetamide have Kᵢ values virtually identical to that of MESC, indicating that the thiol group of MESC does not specifically determine its lower affinity as a polyamine transport inhibitor as compared with DESC. Moreover, these data suggest that additional bulk on the side chain has little influence on the interaction of MESC with the polyamine transporter, in agreement with the observation that large substituents attached to the distal end of a spacer of sufficient length do not notably decrease the affinity of spermidine conjugates for uptake (46).

Unexpectedly, the MESC-cysteamine mixed disulfide (DEASC) blocked putrescine uptake as a mixed competitor/noncompetitor, whereas MESC and DESC behaved like pure competitive inhibitors in that respect. Since the interaction of DESC or MESC with the polyamine carrier was strictly competitive, and because DEASC has a higher affinity than MESC as an inhibitor of polyamine transport, the spermine head and the cysteamine side chain of DEASC might be responsible, respectively, for the competitive and noncompetitive components of its transport inhibition. It is noteworthy that cysteamine and aliphatic monoamines of similar chain length (e.g., n-butylamine and n-pentylamine) have low but significant ability to antagonize putrescine uptake (47), although the mode of inhibition of these compounds has not been reported.

The biochemical properties of DESC clearly show that the binding affinity of an analogue can be dissociated from its ability to serve as a substrate for transport. The large size of DESC cannot be the main factor preventing its carrier-mediated internalization, since MESC was also virtually impermeant. Thus, the attachment of an amido side chain on the spermine backbone would appear to be responsible for the impaired internalization of these analogues. Indeed, N⁴-alkylated spermidine derivatives are much better competitors of spermidine uptake than their N⁴-acyl counterparts in mouse leukemia cells, suggesting that charged secondary amino groups are important for interaction with the polyamine carrier (28). However, the latter argument cannot account for the fact that aliphatic ω,ω-diamines with at least 6 or 7 methylene groups have an affinity comparable with that of spermidine (23, 27, 30, 40, 47). A more likely explanation for the poor affinity of polyamines bearing an acyl side chain might be the steric hindrance due to the amide group, which restricts freedom of rotation around the adjacent carbon and nitrogen atoms. There are indications that cyclic or pseudocyclic conformations of polyamines stabilized by hydrogen bonds might be energetically favored for recognition and/or internalization by the polyamine transporter (23, 27). The formation of such folded conformers would be impaired by close proximity of an amide group to the polyamine chain.

Although a 40-fold molar excess of DESC dramatically reduced the rate of spermidine uptake in ZR-75-1 cells, a low but sustained spermidine accumulation was still observed in the presence of the inhibitor. Furthermore, DESC was slowly inactivated in growth media due to disulfide-disulfide exchange with L-cystine (about 40% degradation after 48 h), although the compound was intrinsically stable in thiol-free aqueous solutions (data not shown). These two factors may largely account for the complete inability of DESC to block polyamine-mediated prevention of growth inhibition by DFMO. Growth inhibition associated with DFMO-induced polyamine depletion in ZR-75-1 cells was completely reversed by concentrations of spermidine as low as 300 nM, i.e., such as those found in human plasma (48–50). The striking efficiency of the transport system to salvage exogenous polyamines in DFMO-treated cells is due to its up-regulation consequent to polyamine depletion (4, 6, 23, 51). These data reinforce the view that cellular uptake of exogenous polyamines is a major factor limiting the efficacy of polyamine biosynthesis inhibitors as antitumor agents in vivo (8–12). Therefore, unless substantial gains in affinity are achieved in the design of competitive inhibitors of polyamine uptake, their efficacy will be kinetically limited by residual transport. In support of this view, mutations of the polyamine transport system make tumor cells much more susceptible to in vivo toxicity.
Polyamine Transport Inhibition by Spermine Analogues

vivo growth inhibition by DFMO than limiting the supply of exogenous polyamines (10). Thus, irreversible inhibition of polyamine transport might present major advantages over pure competition as a strategy to block polyamine uptake. The same rationale has been applied to the inhibition of ornithine decarboxylase, which is better achieved with suicide substrates like DFMO than with competitive inhibitors (52, 53). Moreover, the efficacy of an irreversible inactivation of mammalian polyamine carriers would be advantaged by the apparently long half-life of these transporters (6). The inherent structural features of DESC that confer its high affinity and resistance to uptake should provide a useful framework for the design of potent irreversible inhibitors of polyamine transport.

Since the affinity of MESC thioethers remains virtually unaffected relative to the unconjugated polyamine, MESC-ASIB might serve as a photoaffinity label to detect polyamine-binding proteins, including the polyamine carrier. Experiments are currently conducted with 125I-labeled MES-ASIB to assess its usefulness as a probe to identify the mammalian polyamine transporter. Felschow et al. (54) have described the specific labeling of discrete plasma membrane proteins, using 125I-labeled N-succinimidylsulfosuccinimidylsulfone and N-succinimidylsulfonimidoethyl-spermine as photoaffinity reagents. However, these conjugates are internalized by mammalian cells (54), and MESC-ASIB or similar derivatives could be useful as photoactivatable probes to exclude labeling of intracellular proteins.

The cytotoxicity of high concentrations of DESC and MESC is unlikely to be due to the formation of mixed disulfides between DESC and L-cystine, since MESC was less toxic than DESC, despite the fact that the free thiol group of MESC would make it more reactive toward L-cystine. Moreover, degradation (data not shown) and cytotoxicity could be associated with amine oxidation only in the case of DEASC. Cystamine is a well-known substrate for diamine oxidase (55, 56), and the amine oxidation only in the case of DEASC. Cystamine is a well-known substrate for diamine oxidase (55, 56), and the amine oxidation only in the case of DEASC. Cystamine is a well-known substrate for diamine oxidase (55, 56), and the amine oxidation only in the case of DEASC. Cystamine is a well-known substrate for diamine oxidase (55, 56), and the amine oxidation only in the case of DEASC. Cystamine is a well-known substrate for diamine oxidase (55, 56), and the amine oxidation only in the case of DEASC. 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