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

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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 K_i 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 (K_i = 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 K_i 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 [^{3}H]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-mercap-

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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*-[2,2′-dithio(ethyl,1′-aminoethyl)]-spermine 5-carboxamide; LY, lucifer yellow; ASIB, 1-(*p*-azidosalicylamido)-4-(iodoacetamido)butane; Boc, *tert*-butyl carbonyl; FBS, fetal bovine serum; SAO, serum amine oxidase; HPLC, high performance liquid chromatography; DTT, dithiothreitol; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; CHX, cycloheximide.

toethyl)-spermine 5-carboxamide (MESC; Fig. 1) as a precursor for the synthesis of affinity ligands to label the polyamine carrier. Unexpectedly, we have found that the disulfide form of this compound, or 2,2′-dithiobis(*N*-ethyl-spermine-5-carboxamide) (DESC; Fig. 1) exhibits a much higher affinity than MESC for the polyamine transport system, while being virtually impermeant. In this report, we describe the synthesis and biochemical properties of these compounds and provide evidence that DESC has the attributes expected of a pure and potent competitive antagonist of the polyamine transport system.

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 was performed with a Lichroprep RP-18 C_{18} silica gel column (40–63 μm; BDH, Ville St-Laurent, Québec, Canada) using a gradient of CH_3CN:MeOH:H_2O (25:35:40 to 50:30:20) as eluent. Homogeneity of synthetic products was assessed by thin-layer chromatography performed on 0.20-mm Silica Gel 60 F_{254} plates or 0.25-mm $F_{254}S$ RP-18 reversed phase silica gel plates (E. Merck, Darmstadt, Germany). Fourier transform infrared spectra were obtained on a Perkin-Elmer 1600 spectrophotometer (FTIR series) and were expressed in cm^{-1}. ^1H and ^{13}C NMR spectra were recorded with a Bruker AC/F 300 spectrometer using a Quattro Nucleus probe head tuned to 300.13 MHz (^1H) and 75.47 MHz (^{13}C). NMR samples were prepared by dissolving 10–100 mg of the compounds in 99.8% atom $CDCl_3$ (Omega, Montreal, Québec, Canada) or 99.9% atom D_2O in a 5-mm NMR tube. Chemical shifts (δ, in ppm) were referenced to $CDCl_3$ (7.26 ppm for ^1H and 77.00 ppm for ^{13}C) or to sodium 3-(trimethylsilyl)-1-propane sulfonate in D_2O (0.1169 ppm (s) for ^{13}C). Mass spectra were recorded at the Mass Spectrometry Regional Center (University of Montreal, Québec, Canada) by fast atomic bombardment mass spectrometry (FABMS) or liquid secondary ion mass spectrometry (LSIMS), using a VG AutoSpecQ and a Kratos MS50 TCTA, respectively.

[2,3-^{3}H]Putrescine dihydrochloride (4.1×10^4 Ci/mol) and [1,8-^{3}H] spermidine trihydrochloride (1.5×10^4 Ci/mol) were obtained from DuPont NEN (Lachine, Québec, Canada). [5,8-^{14}C]Spermine tetrahydrochloride (108 Ci/mol) was obtained from Amersham Corp. DFMO was generously provided by the Marion Merrell Dow Research Institute (Cincinnati, OH). Fetal bovine serum (FBS) and Cosmic™ calf serum were purchased from Hyclone (Logan, UT). The heterobifunctional reagent 1-(*p*-azidosalicylamido)-4-(iodoacetamido)butane (ASIB) was obtained from Pierce. Lucifer Yellow (LY) iodoacetamide was purchased from Molecular Probes (Eugene, OR). *ortho*-Phthaldialdehyde was purchased from Fluka (Ronkonkoma, NY), and other reagents for high performance liquid chromatography (HPLC) were obtained from Fisher Scientific (Montreal, Québec, 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 evaporated, the mixture was then dissolved in 350 ml of dry dimethylformamide, and the residual ammonium salt was filtrated, 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^2,N^5-diethylcyanide ornithine, which was subsequently used without further purification. IR (film) ν cm^{-1} 3372 (OH, acid), 2247 (CN); ^1H NMR δ ($CDCl_3$) 1.48 (m, 4H, C$\underline{H}_2$C$\underline{H}_2$CHCOOH), 2.63 (m, 6H, C$\underline{H}_2$N), 2.86 (2t, 4H, C$\underline{H}_2$CN), 3.07 (t, 1H, C$\underline{H}$COOH).

To obtain 5-carboxyspermine (**I**), KOH (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^2,N^5-diethylcyanide ornithine were then added. The resulting mixture was placed under H_2 at 40 p.s.i. in a Burgess-Parr hydrogenator, using 2.09 g (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 (NH_2), no cyanide band; ^1H NMR δ ($CDCl_3$) 1.53 (m, 2H, C$\underline{H}$2CHCOOH), 1.65 (m, 6H, $CH_2C\underline{H}_2CH_2$), 2.51 (m, 4H, C$\underline{H}_2NH_2$), 2.65 (m, 6H, C$\underline{H}_2$NH), 3.09 (t, 1H, C$\underline{H}$COOH).

Synthesis of DESC and N-[2,2′-Dithio(ethyl,1′-aminoethyl)]-spermine 5-Carboxamide (DEASC)—Amine protection of 5-carboxyspermine by *tert*-butyl carbonyl (Boc) groups was performed as described 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-*tert*-butyl dicarbonate. After stirring for 24 h, the solvent was evaporated, 100–150 ml of H_2O were added, and the resulting mixture was chilled at 0 °C. After adjusting the pH at 2.2 with 2 N HCl, the Boc-protected product was extracted with ethyl acetate, dried over anhydrous $MgSO_4$, and purified by C_{18} reversed phase silica gel chromatography, yielding 3.3 g of pure tetra-Boc-5-carboxyspermine (**II**, Fig. 1). Yield of **II** from L-ornithine was 11%. Light yellow solid; IR (film) ν cm^{-1} 3356 (OH, acid), 1682 (C=O, amide). ^1H NMR δ ($CDCl_3$) 1.32 (2 s, 36 H, OC(C$\underline{H}_3$)$_3$), 1.90–1.40 (m, 8H, C$\underline{H}_2CH_2N$), 3.20–2.90 (m, 10H, C$\underline{H}_2$N); ^{13}C NMR δ ($CDCl_3$) 25.62–29.76 (4C, $\underline{C}H_2CH_2NHCOOC(CH_3)_3$, $\underline{C}H_2CH_2NCOOC(CH_3)_3$ and $\underline{C}H_2CHCOOH$), 28.33 (12C, OC($\underline{C}H_3$)$_3$), 37.30 (2C, $\underline{C}H_2NHCOOC(CH_3)_3$), 43.73–46.55 (3C, $\underline{C}H_2NCOOC(CH_3)_3$)), 59.31 and 60.22 (1C, $\underline{C}HCOOH$), 78.92, 79.67, and 80.72 (4C, O$\underline{C}$ (CH_3)$_3$), 155.46 and 156.02 (4C, $\underline{C}OOC(CH_3)_3$), 174.81 (1C, $\underline{C}OOH$). M (for $C_{31}H_{58}O_{10}N_4$) = 646.41; m/z (LSIMS) = 647.42 [$(M + 1)^+$].

Coupling of **II** to cystamine was then performed in two steps based on the method of Venkataraman (16). To a solution of 1.15 g (1.78 mmol) of **II** in 20 ml of dry acetone was added 0.27 ml (1.1 eq, 1.96 mmol) of triethylamine (freshly distilled on KOH) and 361 mg (1.1 eq, 1.96 mmol) of cyanuric chloride, and the reaction mixture was stirred overnight under N_2 to form the corresponding acid chloride. Cystamine dihydrochloride (241 mg; 1.07 mmol) was then suspended in dry triethylamine and added to the acid chloride form of compound **II**, with the resulting triethylamine concentration being at a ≥4-fold excess relative to the latter. After stirring for 12 h, the residual triazine oxide was filtrated, the acetone was evaporated, and the product was extracted with $CHCl_3$, dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The crude compound was then purified by reversed phase C_{18} column chromatography, yielding 0.682 g of 2,2′-dithiobis[*N*-ethyl-(N^1,N^4 N^8,N^{12})-tetra-Boc-spermine-5-carboxamide] (**III**, Fig. 1) and 0.124 g of *N*-[2,2′-dithio(ethyl,1′-aminoethyl)]-N^1,N^4,N^8,N^{12}-tetra-Boc-spermine-5-carboxamide (**IV**, Fig. 1). Yield was 27.2 amd 8.9% for **III** and **IV**, respectively. **III**, yellow oil; IR (film) ν cm^{-1} 1693 (C=O, amide). ^1H NMR δ ($CDCl_3$) 1.38 (s, 36H, ($C\underline{H}_3$)$_3$C), 1.59 (m, 8H, $CH_2C\underline{H}_2CH_2$), 2.53 (t, 1H, CON$\underline{H}$CH_2), 2.78 (t, 2H, C$\underline{H}_2$S), 3.11 (m, 10H, C$\underline{H}_2$NH), 3.51 (m, 2H, NC$\underline{H}_2$CH_2S); ^{13}C NMR δ ($CDCl_3$) 24.40, 24.95, 29.94, and 30.71 (8C, $\underline{C}H_2CH_2NHCOOC(CH_3)_3$ and $\underline{C}H_2CHCONH$), 28.31 (24C, OC($\underline{C}H_3$)$_3$), 37.70–38.42 (6C, $\underline{C}H_2NHCO$), 43.93–45.99 (6C, $\underline{C}H_2NHCOOC(CH_3)_3$), 46.12 (2C, $\underline{C}H_2S$), 62.32 and 62.78 (2C, $\underline{C}HCONH$), 78.96–79.49 (8C, O$\underline{C}$(CH_3)$_3$), 156.00–156.20 (8C, $\underline{C}OOC(CH_3)_3$), 174.81 (2C, CH$\underline{C}$ONH). M (for $C_{66}H_{124}O_{18}N_{10}S_2$) = 1408.85; m/z (FABMS) = 1409.9 [$(M + 1)^+$].

Compound **III** (215 mg in MeOH) was then deprotected by the addition of 1 ml of 3 N HCl, which brought the pH from 6.0 to ~0.5. After stirring vigorously for 15 h, the solvent was dried out *in vacuo*, and the resulting compound was purified by cation exchange chromatography with a Dowex-50W-X4 column (dry mesh: 100–200; Sigma) pre-equilibrated with H_2O and successively washed with H_2O, 1 N HCl, 2 N HCl, 4 N HCl, and 6 N HCl. Ninhydrin-positive fractions eluted with 6 N 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 ($CDCl_3$) 1.62 (m, 2H, C$\underline{H}_2$CHCONH), 1.97–1.80 (m, 6H, $CH_2C\underline{H}_2CH_2$), 2.74 (t, 2H, C$\underline{H}_2$S), 2.92 (m, 10H, C$\underline{H}_2$NH), 3.46 (dt, 2H, C$\underline{H}_2CH_2$S), 3.84 (t, 1H, C$\underline{H}$CONH); ^{13}C NMR δ (D_2O), 23.94, 26.33, 26.38, and 29.58 (8C, $\underline{C}H_2CH_2NH_2$, $\underline{C}H_2CH_2NH$ and $\underline{C}H_2CHCONH$), 38.69 (2C, $\underline{C}H_2S$), 39.19 (4C, $\underline{C}H_2NH_2$), 40.75 (2C, $\underline{C}H_2NHCO$), 46.39, 47.23, and 49.44 (6C, $\underline{C}H_2NH$), 62.83 (2C, $\underline{C}HCONH$), 170.28 (2C, $\underline{C}O$). M (for $C_{26}H_{60}O_2N_{10}S_2$) = 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 DEASC (**VI**, Fig. 1). Yellow solid; m.p. 50–54 °C; b.p. 109 °C. ^1H NMR δ ($CDCl_3$) 1.89 (m, 2H, C$\underline{H}_2$CHCONH), 2.10–2.29 (m, 6H, $CH_2C\underline{H}_2CH_2$), 3.04 (t, 2H, CONHCH_2C$\underline{H}_2$S), 3.19 (t, 2H, SSC$\underline{H}_2CH_2NH_2$), 3.25 (m, 10H, C$\underline{H}_2$NH), 3.51 (t, 2H, SSCH_2C$\underline{H}_2NH_2$), 3.78 (m, 2H, CONHC$\underline{H}_2CH_2$S), 4.11 (t, 1H, C$\underline{H}$CONH); ^{13}C NMR δ (D_2O), 23.96, 26.38, and 29.59 (4C, $\underline{C}H_2CH_2NH_2$, $\underline{C}H_2CH_2NH$, and $\underline{C}H_2CHCONH$), 36.21 (1C, S$\underline{C}H_2CH_2NH_2$), 38.37 (1C, CONHC$H_2\underline{C}H_2$S), 39.19 (2C, $CH_2CH_2\underline{C}H_2NH_2$), 40.49 and 40.57 (2C, SC$H_2\underline{C}H_2NH_2$ and CONH$\underline{C}H_2CH_2$S), 46.40, 47.24, and 49.45 (3C, $\underline{C}H_2CH_2CH_2NH_2$ and $CH_2NH\underline{C}H_2$), 62.83 (1C, $CH_2\underline{C}HCONH$), 170.33 (1C, $\underline{C}O$). M (for $C_{15}H_{41}ON_6S_2$) = 380.62; m/z (LSIMS) = 381.24. 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 dithiothreitol (DTT) and

incubated for 30 min at 37 °C. The mixture was then loaded on a Dowex 50W-X4 cation exchange column equilibrated with H_2O, and 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*-phthaldialdehyde solution (3.7 mM *o*-phthaldialdehyde; 0.4 M boric acid, pH 10.4; 1% (v/v) MeOH; 0.45% (v/v) 2-mercaptoethanol; 0.03% (w/v) Brij 35) and heating for 20 min at 37 °C and then pooled. The amount of MESC tetrahydrochloride (**VII**, Fig. 1) thus isolated was titrated with 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (17) using either cysteamine or DTT as a standard. The yield of MESC using 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 DESC. Finally, MESC purity was confirmed by ion pair reversed phase HPLC using postcolumn derivatization with *o*-phthaldialdehyde (18). ^{1}NMR δ ($CDCl_3$) 1.91 (m, 2H, $C\underline{H}_2CHCONH$), 2.08–2.24 (m, 6H, $CH_2C\underline{H}_2CH_2$), 2.82 (t, 2H, $CONHCH_2C\underline{H}_2SH$), 3.22 (m, 10H, $C\underline{H}_2NH$), 3.56 (m, 2H, $CONHC\underline{H}_2CH_2SH$), 4.11 (t, 1H, $C\underline{H}CONH$); ^{13}C NMR δ (D_2O), 23.87, 26.28, and 29.49 (4C, $\underline{C}H_2CH_2NH_2$, $\underline{C}H_2CH_2NH$, and $\underline{C}H_2CHCONH$), 25.67 (1C, $\underline{C}H_2SH$), 39.09 (1C, $\underline{C}H_2CH_2SH$), 44.90 (2C, $\underline{C}H_2NH_2$), 46.30, 47.13, and 49.35 (3C, $\underline{C}H_2CH_2CH_2NH_2$ and $CH_2NH\underline{C}H_2$), 62.79 (1C, $CH_2\underline{C}HCO$) and 170.28 (1C, $\underline{C}O$).

Synthesis of Thioether Adducts of MESC with Iodoacetamides—To 1 ml of an extemporaneously prepared, DTT-free solution of MESC (20 mM in H_2O) were added 50 μl of 50 mM Tris-HCl (pH 7.0) and 105 μl of a 40 mM solution of iodoacetamide, LY iodoacetamide, or ASIB in a light-protected microcentrifuge tube, and the mixture was incubated for 2 h at 37 °C. Reaction completion was assessed 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 [^{3}H]spermidine uptake assays conducted as described below. The various DTT-inactivated iodoacetamides had no measurable effect on spermidine transport, as determined in parallel by incubating cells with the same reaction mixture from which MESC was omitted.

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 nM 17β-estradiol, and antibiotics (MEZR medium) (19). CHO-K1 cells were routinely grown in α-minimal essential medium supplemented with 10% Cosmic™ calf serum in a 5% CO_2 humid atmosphere at 37 °C.

Effect of Inhibitors on Cell Proliferation—For growth studies, ZR-75-1 cells were cultured in MEZR medium, or in phenol red-free RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM Hepes, antibiotics, 1 nM 17β-estradiol, 0.5 μg/ml bovine insulin, and 5% (v/v) dextran-coated charcoal-treated FBS (SD medium), as indicated. In some experiments, ZR-75-1 cells were also grown in Richter's improved minimum essential medium supplemented as for SD medium (19). When polyamines or polyamine analogues were added to serum-containing media, 1 mM aminoguanidine was added to inhibit bovine serum amine oxidase (SAO) activity (20). The effect of the transport inhibitors on cell growth was measured by incubating ZR-75-1 cells for 11 days in medium supplemented with antagonist, polyamines, and/or 1 mM DFMO with the addition of fresh medium every other day. Total cellular DNA was then fluorometrically determined with 3,5-diaminobenzoic acid (21).

Polyamine Analysis—ZR-75-1 cells were plated in 100-mm culture dishes at 5×10^5 cells/dish in MEZR medium and grown for 5 days. Fresh MEZR medium containing the indicated concentration of transport antagonist was then added, plus or minus 200 μM cycloheximide (CHX). After 1–6 h of incubation, medium was removed, cell monolayers were rinsed twice with 10 ml of ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) (2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl), and cells were harvested by centrifugation ($2000 \times g$ for 90 s at 4 °C) following a 5–7-min incubation with bovine trypsin/EDTA solution (0.05%/0.02%) in Hanks' balanced salt solution (19). Cell pellets were resuspended in 300 μl of 10% (v/v) trichloroacetic acid or Tris-DTT buffer (50 mM Tris/HCl, 0.1 mM EDTA, 5 mM DTT, pH 7.5) and stored at −20 °C until further analysis. For chromatographic analysis, samples were first quickly thawed and incubated for 15 min at 37 °C. Trichloroacetic acid was then added to DTT-containing samples to a final concentration of 10% (w/v). Samples were dispersed for 2 min in a sonicating water bath, and pelleted in a microcentrifuge for 5 min. The trichloroacetic acid-insoluble pellet was solubilized in 300–500 μl of 1 N NaOH and used to determine protein content (22) using bovine serum albumin (fraction V) as standard. Polyamine content was then determined by ion pair reverse phase HPLC with fluorometric detection after postcolumn derivatization with *o*-phthaldialdehyde as described (18, 19). In this system, putrescine, spermidine, spermine, MESC, DEASC, and DESC were resolved with retention times of 18.5, 31.0, 35.0, 36.5, 37.5, and 44.0 min, respectively.

DESC stability was tested by incubating the compound dissolved (at 50 μM) in PBS or in Richter's improved minimum essential medium containing 10% (v/v) FBS plus or minus 1 mM aminoguanidine in a humid 5% CO_2 atmosphere at 37 °C in the absence of cells. Trichloroacetic acid was added to aliquots of this solution to a final concentration 10% (w/v), and the samples were directly analyzed by HPLC as above.

Determination of Polyamine Uptake Activity—The rate of putrescine and spermidine transport was determined in ZR-75-1 cells incubated in serum-free RPMI 1640 medium as described (23), using [^{3}H]putrescine (30 Ci/mol) and [^{3}H]spermidine (20 Ci/mol), respectively, as substrates for a 20-min assay period. Spermine uptake was similarly determined, using 1 μM [^{14}C]spermine (32 Ci/mol) as substrate. Uptake activity was expressed per amount of DNA as fluorometrically determined using 3,5-diaminobenzoic acid (21). The time course of intracellular spermidine accumulation was similarly determined by incubating ZR-75-1 cells with transport inhibitors dissolved in MEZR medium containing 5 μM [^{3}H]spermidine in the presence or absence of 200 μM CHX.

For the 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 mM $CaCl_2$, 0.41 mM $MgSO_4$, 103 mM NaCl, 5.7 mM KCl, 1.1 mM D-glucose) containing 5 μM [^{3}H]spermidine (20 Ci/mol). Cell cultures were then washed twice with 1 ml of PBS containing 5.7 mM *sym*-norspermidine. 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 (24). Nonspecific binding of radioactive substrate was similarly determined in parallel for both cell lines after a 15-s incubation with 400 μl of ice-cold uptake solution.

Kinetic parameters of polyamine transport were determined by Lineweaver-Burke analysis of uptake activity in the presence of 0.3 μM [^{3}H]putrescine or 0.1 μM [^{3}H]spermidine plus increasing concentrations of nonradioactive substrate. For competitive inhibitors, K_i 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,

$$v = \frac{V_{max}}{\frac{K_m}{s}\left(1 + \frac{i}{K_i}\right) + \left(1 + \frac{i}{K_i'}\right)} \quad \text{(Eq. 1)}$$

where v, s, and i are the transport velocity, substrate concentration, and inhibitor concentration, respectively, was used to calculate the inhibition constants for inhibitor-carrier complex formation (K_i) and carrier-inhibitor-substrate complex formation (K_i') (25). Alternatively, the value of K_i was estimated from the intersection of equations of v^{-1} *versus* i at two substrate concentrations (25).

Statistical Analysis—The statistical significance of differences between means was assessed by unpaired Student's t tests. Unless otherwise indicated, results are expressed as means ± S.D. of determinations from triplicate cell cultures.

RESULTS

Design and Synthesis of DESC, DEASC, and MESC—Our original rationale for synthesizing MESC (**VII**, Fig. 1) was to generate an affinity reagent with a thiol side chain that could be derivatized with fluorescent or radioactive sulfhydryl reagents to label the polyamine transporter. The scheme used to prepare MESC involved the coupling of a cystamine bridge through amide bonds with two Boc-protected 5-carboxyspermine molecules to form DESC after removal of the Boc groups (**V**, Fig. 1), followed by reduction of the DESC disulfide bridge. A small amount (10–15%) of the mixed MESC-cysteamine disulfide (DEASC, **VI**; Fig. 1) was also generated in the coupling process. Complete separation of DEASC from DESC on a preparative basis proved to be difficult even using ion exchange

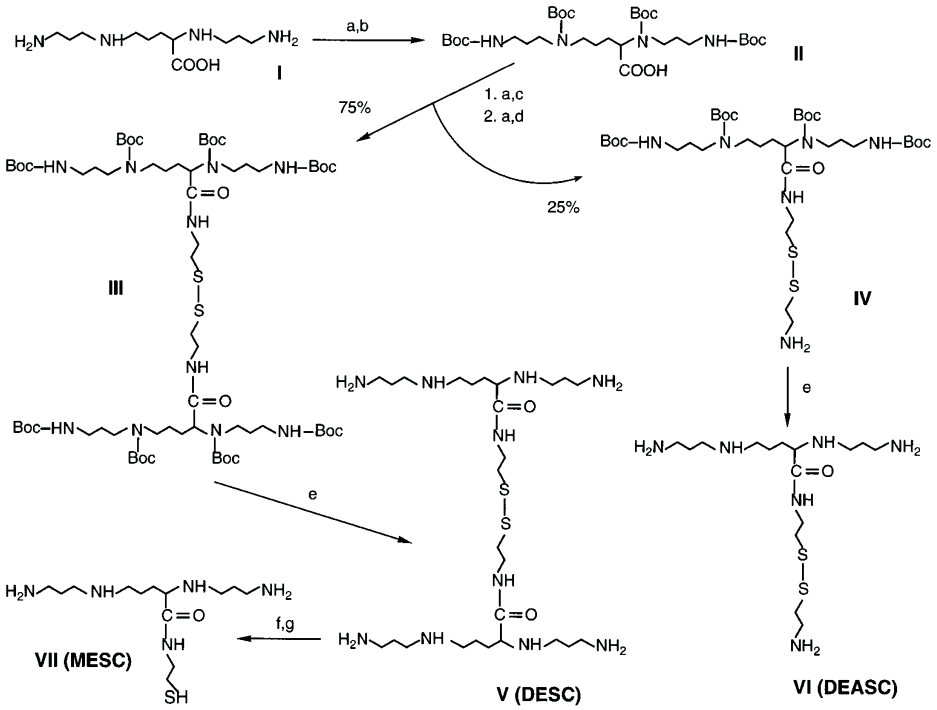

FIG. 1. **Scheme for the synthesis of DESC, DEASC, and MESC.** Details of the synthesis are as given under "Experimental Procedures." *a*, triethylamine; *b*, di-*tert*-butyl dicarbonate; *c*, cyanuric chloride; *d*, cystamine dihydrochloride; *e*, 3 N HCl; *f*, dithiothreitol; *g*, 50 mM sodium phosphate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

chromatography (data not shown); thus, most DESC preparations contained a small amount (1–2%) of DEASC after reversed phase liquid chromatography on C_{18} silica gel. DESC and DEASC were stable for months at −20 °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 [^{14}C]spermine transport in ZR-75-1 cells, with a K_i value about 5- and 16-fold lower than that of DEASC and MESC, respectively (Fig. 2). Moreover, the ability of spermine to compete against [^{3}H]putrescine and [^{3}H]spermidine uptake was in fact only about 7-fold higher than for DESC (Fig. 3). DESC (Fig. 4*A*) and MESC (data not shown) were pure competitive inhibitors of [^{3}H]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. 4*B*). Table I summarizes the K_i 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, K_i 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 K_i roughly equal to their K_m as substrate. In CHO cells, DESC and MESC competitively inhibited spermidine uptake, with K_i 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,

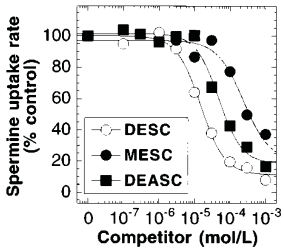

FIG. 2. **Inhibition of [^{14}C]spermine transport by MESC, DESC, and DEASC in human ZR-75-1 breast cancer cells.** The rate of spermine uptake was measured in ZR-75-1 cells grown as monolayers in 24-well culture plates in the presence of the indicated concentrations of DESC (○), MESC (●), and DEASC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

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 ^{125}I-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 K_i toward spermidine uptake for the three conjugates studied. In the case of MESC-ASIB, K_i 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,

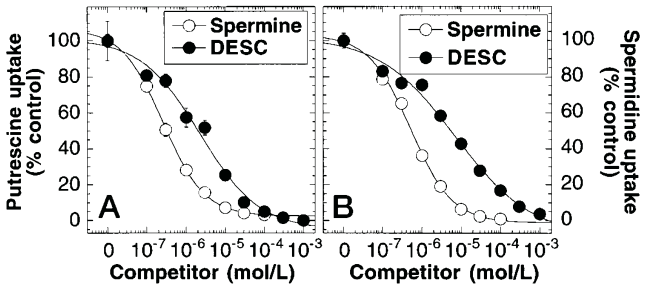

FIG. 3. **Inhibition of [^{3}H]putrescine and [^{3}H]spermidine uptake by spermine and DESC in ZR-75-1 cells.** The rate of spermidine uptake was measured in ZR-75-1 cells grown as monolayers in 24-well culture plates in the presence of the indicated concentrations of spermine (○) and DESC (●), using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

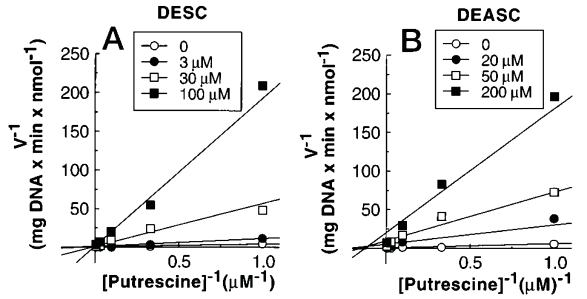

FIG. 4. **Lineweaver-Burk analysis of putrescine transport inhibition by DESC and DEASC in ZR-75-1 cells.** The rate of [^{3}H]putrescine uptake was determined in ZR-75-1 cell cultures with increasing concentrations of substrate in the presence of 0 (○), 3 (●), 30 (□), or 100 μM DESC (■) (*A*) or in the presence of 0 (○), 20 (●), 50 (□), or 200 μM DEASC (■) (*B*).

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, but not 50 μM, DESC. These levels represent only about 1.5% of the accumulation measured for spermidine in ZR-75-1 cells under identical conditions (see below). Moreover, CHX, which is known to up-regulate polyamine uptake by preventing the synthesis of a polyamine-induced transport repressor (23, 35), did not enhance DESC internalization, in marked contrast with its effect on spermidine accumulation under similar conditions (*cf.* Fig. 6*B*) (23). Likewise, MESC was accumulated to measurable levels only when present at 200 μM (Table II). Thus, neither DESC or MESC appears to be used as substrate for the polyamine transport system, despite the high potency of the former as an uptake antagonist.

Effect of DESC and MESC on Intracellular Polyamine Accumulation—To further evaluate the capacity of DESC and MESC to block polyamine uptake, the time course of internalization of radiolabeled spermidine was determined in ZR-75-1 cells incubated for up to 6 h in the presence of the impermeant agonists. As shown in Fig. 6*A*, steady-state [^{3}H]spermidine accumulation in the absence of competitor abruptly reached a near plateau after about 1 h, which results from the induction

TABLE I
K_i values of inhibition of diamine and polyamine transport by MESC, DESC, and DEASC in ZR-75–1 cells

Compound	K_m or K_i		
	Putrescine	Spermidine	Spermine
		μM	
Putrescine	3.7 ± 0.4[a]	125 ± 29[a]	0.23 ± 0.13[a,b]
Spermidine	0.23 ± 0.05[a]	0.49 ± 0.15[a]	0.37 ± 0.09[a]
Spermine	0.33 ± 0.02[a]	ND[c]	0.20 ± 0.06[b]
DESC	1.6 ± 0.5[b]	2.7 ± 1.1[b]	5.0 ± 0.7[b]
MESC	22 ± 3[b]	ND	80 ± 31[b]
DEASC	5.3 ± 0.6 (K_i)[d]	ND	16 ± 3[e]
	4.1 ± 0.5 (K_i')		

[a] From Ref. 23.
[b] This work; mean ± S.D. of triplicate determinations from two to four different experiments.
[c] ND, not determined.
[d] Values of inhibition constants for carrier-inhibitor complex formation (K_i) and for carrier-inhibitor-putrescine complex formation (K_i') assuming a mixed competitive/noncompetitive model (*cf.* "Experimental Procedures"): mean ± S.D. of triplicate determinations at three inhibitor concentrations.
[e] Value of K_i determined at two different substrate concentrations for a series of increasing inhibitor concentrations (25).

R	Name	K_i (µM)
H	MESC	33.6 ± 7.2
–CH$_2$–C(=O)–NH$_2$	MESC-acetamide	48.9 ± 9.1
–CH$_2$–C(=O)–NH– (sulforhodamine)	MESC-LY	44.1 ± 8.8
–CH$_2$–C(=O)–NH– (HO, NH–C(=O), N$_3$)	MESC-ASIB[a]	18.3 ± 8.2

[a] Value for K_i is likely underestimated due to partial irreversible inhibition of the polyamine transporter (cf."Results").

FIG. 5. **Structure of MESC thioether derivatives and their K_i 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 [^{3}H]spermidine as substrate. K_i values are given as the mean ± S.D. of triplicate determinations from two or three experiments.

of feedback transport inhibition (23). MESC and DESC decreased the initial rate of spermidine uptake according to their respective potencies as competitive antagonists. Interestingly, spermidine accumulation in the presence of either inhibitor followed a pattern similar to that of control cells, *i.e.* a rapid phase during the first 60 min followed by a much slower rate of accumulation thereafter, which was nearly independent of antagonist concentration. Nevertheless, even a 40-fold excess of the most potent antagonist (*i.e.* 200 μM DESC) decreased *net* spermidine accumulation by only 50% after 6 h. As previously observed (23), CHX abolished the induction of feedback transport inhibition, resulting in a 4-fold increase in spermidine accumulation after 4 h (Fig. 6*B*). Protein synthesis inhibition also enhanced spermidine accumulation in DESC-treated cells, a finding consistent with the onset of substantial feedback transport repression induced by low levels of internalized substrate. Thus, in the absence of the feedback mechanism, DESC (200 μM) decreased net [^{3}H]spermidine accumulation by 80–

TABLE II
Intracellular accumulation of DESC and MESC in ZR-75–1 cells

ZR-75–1 cells were incubated for 1 or 6 h in MEZR medium 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.

Addition	Time	Polyamine intracellular contents			
		Spermidine	Spermine	DESC	MESC
	h	*nmol/mg protein*			
Control	1	0.69 ± 0.08	8.22 ± 0.48		
	6	0.91 ± 0.07[a]	9.16 ± 0.13		
50 μM DESC	1	0.81 ± 0.14	8.27 ± 0.81	<0.01	<0.01
	6	0.73 ± 0.11	8.60 ± 0.29	<0.01	<0.01
200 μM DESC	1	0.79 ± 0.11	8.77 ± 0.79	<0.01	<0.01
	6	0.76 ± 0.11	8.66 ± 0.26	0.12 ± 0.01	<0.01
200 μM DESC and CHX	1	0.75 ± 0.04	9.57 ± 0.31	<0.01	<0.01
	6	0.70 ± 0.03	9.55 ± 0.13	0.10 ± 0.01	<0.01
50 μM MESC	1	0.95 ± 0.11	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[a]	8.93 ± 0.53	<0.01	0.020 ± 0.005
	6	0.81 ± 0.15	8.32 ± 0.43	<0.01	0.13 ± 0.06

[a] Significantly different ($p < 0.05$) from control value at time = 1 h.

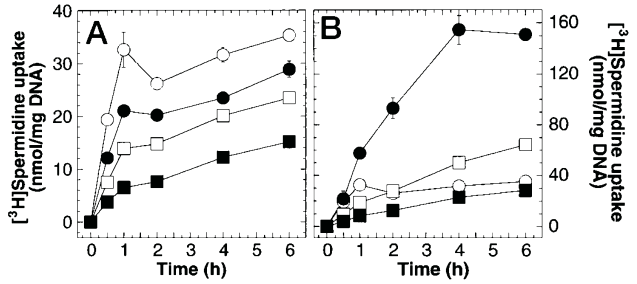

FIG. 6. **Effect of DESC and MESC on the intracellular accumulation of [^{3}H]spermidine in ZR-75-1 cells.** *A*, at time zero, 5 μM [^{3}H]spermidine was added to ZR-75-1 cell cultures grown in 24-well plates (1 ml/well), in the presence of 200 μM MESC (●), 50 μM DESC (□), or 200 μM DESC (■), and the accumulation of radiolabeled spermidine was determined after the indicated interval. Control cells (○) received vehicle only. *B*, same as *A*, except that 200 μM CHX was added at time zero in the presence of 0 (●), 50 (□), or 200 μM DESC (■). Data are the mean ± S.D. of triplicate determinations.

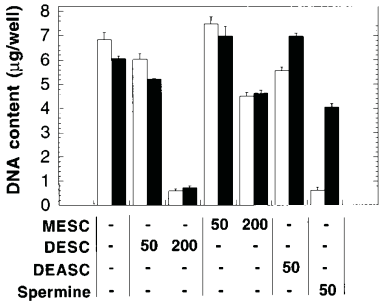

FIG. 7. **Effect of spermine, MESC, DESC, and DEASC on ZR-75-1 cell proliferation.** Cells were incubated for 11 days in MEZR medium with the indicated concentration of spermine, DESC, MESC, or DEASC in the presence (*shaded bars*) or absence (*plain bars*) of 1 mM aminoguanidine, and DNA content per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

85% after 6 h and to a level lower than that found in control cells with fully repressed transport.

Effect of DESC, DEASC, and MESC on Cell Proliferation—Due to the analogy of the novel transport antagonists with spermine, significant cytotoxicity would be expected from these compounds, as for the parent molecule. The marked toxicity of low ($<10^{-3}$ M) spermine concentrations in biological media mostly results from catabolism by SAO, which generates a dialdehyde, acrolein, and H_2O_2 as deleterious products and can be irreversibly inhibited by carbonyl reagents such as aminoguanidine (20). Growth inhibition by DESC, MESC, and DEASC was thus evaluated in ZR-75-1 cells grown in the absence and presence of 1 mM aminoguanidine. Aminoguanidine alone had a slight inhibitory effect on ZR-75-1 cell growth as previously observed (19) (Fig. 7). Although DESC was mildly growth inhibitory at 50 μM, there was a dramatic, aminoguanidine-resistant increase in toxicity at 200 μM. In contrast, spermine was acutely cytotoxic at 50 μM, an effect that was partly prevented by aminoguanidine. MESC was much less toxic than its dimer, with a 35% decrease in cell mass at 200 μM, which was not blocked by aminoguanidine. On the other hand, 50 μM DEASC caused a 20% inhibition of cell number, which could be completely prevented by the amine oxidase inhibitor. Thus, DESC, and to a much lesser degree its thiol monomer MESC, are cytotoxic at high concentrations toward breast cancer cells via a mechanism that does not involve SAO. Weak growth inhibition caused by the mixed MESC-cysteamine disulfide, however, apparently involved degradation by a 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-

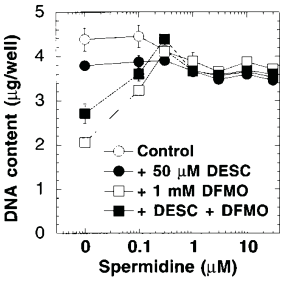

FIG. 8. **Effect of DESC on the reversal of DFMO-induced growth inhibition by exogenous spermidine in ZR-75-1 cells.** Cells were incubated for 11 days in SD medium with the indicated concentrations of spermidine in the presence of 50 μM DESC (●), 1 mM DFMO (□), the combination thereof (■), or in the absence of drugs (○). Data are the mean ± S.D. of determinations from triplicate cultures.

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 paraquat (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 K_i 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 K_i 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 K_i 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 (pK_a ~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-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine, 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 K_i 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, N^4-alkylated spermidine derivatives are much better competitors of spermidine uptake than their N^4-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 α,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 import 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 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 ^{125}I-labeled MESC-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 ^{125}I-labeled N^1-azidosalicylamido-norspermine and N^4-azidosalicylamidoethyl-spermidine as photoaffinity reagents. However, these conjugates are internalized by mammalian cells (54), and MESC-ASIB or similar derivatives could be useful as photoactivable 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 half-cystamine side chain of DEASC could be susceptible to attack by that serum enzyme. However, causes for the apparent lack of degradation of MESC and DESC by SAO are less clear, since, like spermine, both compounds have free aminopropyl ends, which are the basic substrate requirements for SAO (20, 57). The presence *per se* of a bulky side group does not prevent the attack of polyamine conjugates by SAO (18). On the other hand, DESC and MESC cytotoxicity could result from their slow but detectable internalization. The very low rate of MESC and DESC accumulation could result in part from endocytosis as well as diffusion, which becomes increasingly important at concentrations $>10^{-4}$ M for compounds like spermine (58, 59). Nevertheless, the present data clearly show that DESC has remarkably low toxicity in comparison with spermine. Thus, the basic features of this molecule should be useful for the design of potent transport inhibitors endowed with low cytotoxicity.

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