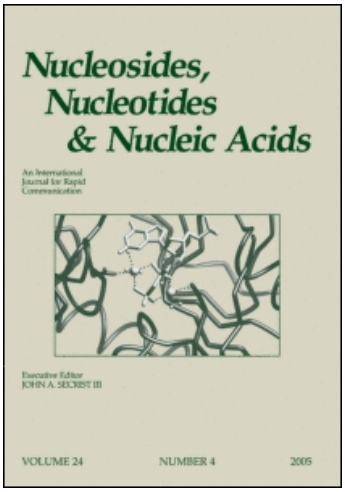

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Conjugates of Fluorescein and Saenta (5'-*S*-(2-Aminoethyl)-N^6-(4-nitrobenzyl)-5'-thioadenosine): Flow Cytometry Probes for the *ES* Nucleoside Transporter Elements of the Plasma Membrane

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CONJUGATES OF FLUORESCEIN AND SAENTA (5'-*S*-(2-AMINOETHYL)-N^6-(4-NITROBENZYL)-5'-THIOADENOSINE): FLOW CYTOMETRY PROBES FOR THE *ES* NUCLEOSIDE TRANSPORTER ELEMENTS OF THE PLASMA MEMBRANE*

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Abstract: SAENTA was linked to the C-5 or C-6 positions of fluorescein through several structures to form conjugates that were bound tightly to plasma membrane sites associated with *es* nucleoside transport activity. The conjugates imparted fluorescence to cells that expressed *es* nucleoside transport activity and served as *es*-selective plasma membrane stains suitable for flow cytometry. Prior treatment of *es*-expressing cells with nitrobenzylthioinosine prevented fluorescent staining with the conjugates. Seven SAENTA-fluorescein conjugates served as flow cytometric stains with high affinities for *es* sites, despite substantial differences in the SAENTA-fluorescein linkage structures.

INTRODUCTION

Equilibrative (facilitated diffusion) and Na^+-dependent, concentrative transport systems are the primary means by which physiological nucleosides and many of their analogues traverse the plasma membrane in mammalian cells[1-4]. At least two equilibrative nucleoside transport (NT) systems mediate the entry and exit of

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* Dedicated to the memory of Professor Roland K. Robins

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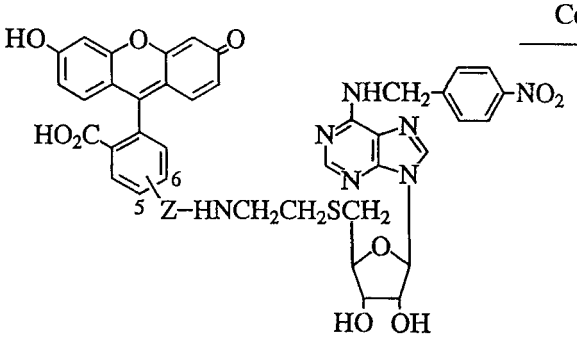
ribosides and deoxyribosides of the physiological purines and pyrimidines, and of related synthetic bases[3-7], and are referred to as *es*[8] and *ei* NT systems[7]. Of these, the best-studied is the widely distributed *es* nucleoside transporter, which is highly sensitive to inhibition by NBMPR (S^6-(4-nitrobenzyl)-6-thioinosine) and by N^6-(4-nitrobenzyl)adenosine derivatives[9]. The *ei* NT system is relatively insensitive to inhibition by NBMPR, but is sensitive to inhibition by dipyridamole, which also inhibits the *es* system[10]. The *es* NT system is expressed in human erythrocytes[4] and in many cultured cell lines, including S49 mouse lymphoma cells[11]. The *ei* system is also expresed in various cultured cell lines including Walker 256 carcinoma cells[6], Novikoff hepatoma cells[12] and L1210 leukemia cells[7]. Two or more NT systems may be expressed in cells, as in the instance of clonal lines of L1210 mouse leukemia cells that express both *es* and *ei* equilibrative NT systems, together with a Na^+-dependent, concentrative NT system designated *cif*[7]. The *cif* transporter, which has been recognized in mouse and rat cells[7,13-15], is insensitive to the potent inhibitors of *es* NT processes, NBMPR and dipyridamole, and accepts formycin B as a permeant. The L1210/MC5-1 clonal line, which was derived from an L1210 wild-type line by mutation and selection procedures, lacks the *ei* transporter and expresses only the *es* and *cif* NT systems[16].

The well-studied inhibitor of the *es* NT process, NBMPR, is reversibly bound by *es* transporter molecules with high affinity (K_d, 0.1-1.0 nM)[1-4]. In many cell types, inhibition of the *es* NT system by NBMPR correlates with NBMPR-site occupancy[11-16], and the abundance of high affinity NBMPR-binding sites appears to be a measure of *es* transport capacity[17]. Photoaffinity labelling with [^{3}H]NBMPR has identified an intrinsic plasma membrane glycoprotein as the *es* nucleoside transporter[18,19]. The work of Wiley *et al.*[20] and White *et al.*[21] indicates that the *es* nucleoside transport capacity is a determinant of the rate of arabinosylcytosine accumulation in fresh human leukemia cells.

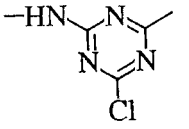
The tightly-bound fluorescent ligands for the *es* nucleoside transporter described here provide a means for the flow cytometric detection and quantitation of the *es* nucleoside transporter. Shohami and Koren[22] described the first fluorescent probe designed for the *es* transporter, *S*-(*N*-dansyl-aminoethyl)-6-mercaptoguanosine; however, use of that compound as a flow cytometric stain has not been reported. This report concerns a set of SAENTA-fluorescein conjugates, Compounds **1-7**, the structures of which are shown in Table 1. Earlier reports showed that (i) Compound **1** (5-(SAENTA-x2)-fluorescein (or 5-Sx2-F (see Table 1 for abbreviations)) is a tightly-bound, competitive inhibitor of arabinosylcytosine transport (IC_{50}, 40 nM) and of NBMPR binding in human RC2a leukemia cells[23],

TABLE 1. SAENTA-fluorescein conjugates: structures and properties

R_F values are from thin-layer silica gel chromatograms developed in acetonitrile/50 mM ammonium bicarbonate (4:1, v/v). H.p.l.c. retention time (R_T) values refer to isocratic elution (3 ml/min) at 40°C with methanol-buffer solution (methanol in 10 mM sodium phosphate buffer, pH 7.0 (9:11, v/v)) on a Partisil 10 ODS-3 M9 (50 cm) column (Whatman, Clifton, NJ).



Compounds 1 - 7

Compound	Linkage(Z)*
1, 2	-NHCS-
3, 4	-CONH(CH$_2$)$_5$CO-
5, 6	-CO-
7	

Compound		Yield (%)	T.l.c. R_F	H.p.l.c. R_T(min)	FABMS (m/z)	
No.	Abbrev.				Calcd. (M^+)	Found ($M^+ + H$)
1	5-Sx2-F	60	0.77	18.4	850	851
2	6-Sx2-F	70	0.78	11.1	850	851
3	5-Sx8-F	80	0.74	15.9	932	933
4	6-Sx8-F	80	0.75	10.7	932	933
5	5-Sx1-F	55	0.76	13.4	819	820
6	6-Sx1-F	50	0.77	9.7	819	820
7	5-Sx4-F	50	0.80	41.7	919	919**

* Group Z was linked to the fluorescein C-5 position except in Compounds **2, 4,** and **6,** in which Z was C-6-linked.

** In this instance, an M^+ peak was observed.

and (ii) Compounds **1** and **3** (5-Sx8-F) serve as flow cytometric stains for these cells[24]. The present study compared as flow cytometric stains Compounds **1** and **3** and these additional SAENTA-fluorescein conjugates: 6-Sx2-F (Compound **2**), 6-Sx8-F (Compound **4**), 5- and 6-Sx1-F (Compounds **5** and **6**), and 5-Sx4-F (Compound **7**). This study compared the influence of linkage variations and of attachment at the C-5 and C-6 positions of fluorescein on the performance of the SAENTA-fluorescein conjugates as flow cytometric stains for the *es* transporter in L1210/B23.1 cells, a clonal line in which only *es* NT activity is expressed[25].

EXPERIMENTAL

Materials. SAENTA phthaloylhydrazide salt and NBMPR were synthesized as previously described[26,27]. Fluorescein derivatives were purchased from Molecular Probes Inc., Eugene, OR, and fluorescein was from Sigma, St. Louis, MO. Silica gel 60 F_{254} powder for preparative t.l.c. and analytical silica gel t.l.c. plates (0.25 mm, 20 x 20 cm plastic plates) were from Merck, Rahway, NJ. Deuterated dimethylsulfoxide containing 0.03% tetramethylsilane as an internal standard was purchased from Aldrich Chemical Co., Milwaukee, WI. Cell culture materials were purchased from Gibco, Burlington, Ont., Canada. Silicone 550 oil was from Dow Corning, Mississauga, Ont., Canada. Tritiated nucleosides were purchased from Moravek Biochemicals, Brea, CA, and after storage were purified by h.p.l.c. on a Partisil 10 ODS-3 M9 (25 cm) column (Whatman, Clifton, NJ) eluted with methanol-water solutions.

Analytical Methods. U.v. spectra were recorded for Compounds **1-7** in methanol-buffer solution (methanol added to 10 mM sodium phosphate buffer, pH 7.0 (7:13, v/v)) on a Hitachi 3200 spectrophotometer and fluorescence spectra were obtained with a Perkin Elmer LS50 luminescence spectrophotometer. Excitation spectra were recorded in the 400-510 nm range with an emission wavelength of 520 nm. Uncorrected emission spectra were recorded in the 505-550 range, with excitation at 488 nm, with slit widths for excitation and emission set at 2.5 and 5.0 nm, respectively. In comparisons of fluorescence output, equiabsorbing solutions of compounds at concentrations $\leq 1\ \mu M$ were excited at 488 nm, emission energy was collected through a 530/30 nm band pass filter and areas under the fluorescence intensity curves obtained in the 510-630 nm range were taken to represent the relative fluorescence output. Fluorimetric determination of equilibrium concentrations of unbound SAENTA-fluorescein conjugates in flow cytometric assay samples was conducted with a Perkin Elmer model MPF-4 spectrofluorimeter. High resolution proton NMR spectra were recorded with a Bruker AM300

spectrometer for solutions of compounds in deuterated dimethylsulfoxide containing tetramethylsilane as an internal standard by the NMR Laboratory, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. FABMS (fast atom bombardment mass spectrometry) was performed with a Kratos MS-50 mass spectrometer, in dithiothreitol/dithioerythritol (6:1, w/w) matrix by the Mass Spectrometry Laboratory, Department of Chemistry, University of Alberta.

Synthesis and purification of SAENTA-fluorescein conjugates. SAENTA phthaloylhydrazide salt was treated with amine-reactive fluorescein derivatives in the presence of triethylamine in the dark. Products were isolated by preparative t.l.c. and purified by reversed phase h.p.l.c., which resolved 5- and 6-fluorescein product mixtures. The following preparation of 6-Sx2-F (Compound **2**) is representative of the SAENTA-fluorescein conjugate preparations in this study. A solution of SAENTA phthaloylhydrazide (3.1 mg, 0.005 mmol), 6-fluorescein isothiocyanate (3.9 mg, 0.01 mmol) and triethylamine (5 μl, 0.05 mmol) in methanol (20 ml) was stirred at 37°C for 2 h in the dark. The reaction mixture was concentrated under reduced pressure at 22°C and the residue was subjected to preparative silica gel t.l.c. with acetonitrile/0.05 M ammonium bicarbonate - 0.05 M ammonium chloride solution (4:1, v/v). The fluorescent material with R_F 0.78 was recovered from chromatogram scrapings by extraction with methanol (2 x 75 ml). The extract was concentrated and subjected to reversed phase h.p.l.c. on a Dynamax-300A C_{18} column (21.4 x 250 mm) (Rainin Instruments, Woburn, MA) by isocratic elution with a solution of methanol and 10 mM sodium phosphate buffer, pH 7.0 (4:5, v/v), at 3 ml/min. Fractions with R_T of 40 min (major peak) were pooled, dried under reduced pressure at 40°C and the residue was extracted with methanol (3 x 2 ml). Methanol extraction of the residues from the combined methanol extracts was repeated twice with efforts to minimize extract volumes. Distilled water (3 ml) was added to the final methanol extract (1 ml) and Compound **2** crystallized as an orange powder that was washed with distilled water and dried under reduced pressure at 78°C (yield, 2.7 mg (70%)). Other SAENTA-fluorescein conjugates, Compounds **1** and **3** to **7** (Table 1), were prepared similarly; the fluorescein derivatives listed in Table 2 were reacted with SAENTA phthaloylhydrazide in the preparation of those conjugates. Physical data for Compounds **1-7** are presented in Table 1. Absorption maxima in methanol-buffer solution are reported in Table 3.

Cell culture. Stocks of L1210/B23.1 and L1210/MA-27.1 cells[25,28] were maintained in exponential growth (0.5 x 10^5 - 5 x 10^5 cells/ml) in RPMI 1640

TABLE 2. Fluorescein derivatives used to prepare SAENTA-fluorescein conjugates

Compounds	Fluorescein derivatives*
1,2	5- and 6-Fluorescein isothiocyanates (FITC I,II)
3,4	6-(Fluorescein-5(and-6)-carboxamido)hexanoic acid, succinimidyl esters (SFX)
5,6	5(and 6)-Carboxyfluorescein, succinimidyl esters (CFS)
7	5-(4,6-Dichlorotriazinyl)aminofluorescein (DTAF)

* Molecular Probes Inc., Eugene, OR.

TABLE 3. SAENTA-fluorescein conjugates: Absorption spectra
Spectral data were obtained from solutions of the conjugates in methanol-buffer solution (methanol added to 10 mM sodium phosphate buffer, pH 7.0 (7:13, v/v). The molar extinction coefficients (parentheses) are means of three separate determinations, except the data for Compounds **6** and **7**, which represent two determinations each.

Compound	Absorption maxima (nm) and molar extinction coefficients			
1	240 (57,600)	266 (48,800)		496 (67,600)
2	240 (52,900)	271 (47,400)		498 (63,600)
3	240 (48,600)	265 (37,400)	321 (11,200)	498 (69,100)
4	239 (50,500)	261 (36,700)		498 (70,700)
5	240 (52,800)	264 (41,100)	322 (12,800)	498 (70,000)
6	239 (46,300)	261 (36,500)		498 (63,800)
7	237 (61,800)	272 (53,200)		495 (70,700)

medium with 10% horse serum at 37°C in a humidified atmosphere of 5% CO_2 in air.

Flow cytometry. For flow cytometry, cells were washed once at 22°C and suspended at 1.6 x 10^6 cells/ml in phosphate-buffered saline[29], pH 8.0 (PBS). Cell-associated fluorescence was measured with a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 mW-argon laser (Faculty of Medicine Flow Cytometry Facility, University of Alberta). The stained cells were excited at 488 nm and fluorescence energy was collected through a

530-nm band pass filter. A four-decade logarithmic amplification across 1024 channels was employed for fluorescence histogram displays. Forward scatter and right-angle (side) scatter parameters were used to gate-out fluorescence signals from cell debris. In each assay, 14,000 cells were analyzed from suspensions of 4×10^5 cells/ml that had been incubated with the SAENTA-fluorescein conjugates for 45 min at 22°C. A portion of each assay mixture was cleared of cells by centrifugation immediately following flow cytometric analysis and concentrations of the SAENTA-fluorescein conjugates in the medium were determined by fluorimetry.

Inhibition of [^{3}H]NBMPR binding. For assays of [^{3}H]NBMPR binding, cells were washed once in RT medium (RPMI 1640 medium without serum or bicarbonate, but containing 20 mM Hepes buffer (pH 7.4)) and resuspended in the same medium at 2×10^6 cells/ml. Equilibrium binding of [^{3}H]NBMPR to L1210/B23.1 cells was measured by a previously described procedure[11] in which cells were incubated for 45 min at 22°C with graded concentrations (0.05 nM-1.0 nM) of [^{3}H]NBMPR in the presence or absence of a competing SAENTA-fluorescein conjugate at several concentrations. The assays were conducted in triplicate in RT medium, and assay mixtures (1.0 ml) were contained in 1.5-ml microcentrifuge tubes. After the above-specified incubation, 200 μl of a silicone 550 oil/paraffin oil mixture (ρ, 1.03 g/ml)[11] were added to each assay mixture, cells were then centrifugally pelleted under the oil (16,000 x g, 30s), and supernatants were sampled for measurement of ^{3}H content. After assay tubes were washed with water above the oil, the oil and rinsings were removed by aspiration, and the cells were lysed in 5% Triton X-100 for assay of the [^{3}H]NBMPR content of cell pellets by liquid scintillation counting. For determination of nonspecific binding of [^{3}H]NBMPR, assay mixtures contained 10 μM nonisotopic NBMPR. Mass law analysis of binding data was performed with the Ligand computer program[30].

RESULTS

Synthesis and characterization of SAENTA-fluorescein conjugates. The reaction of SAENTA phthaloylhydrazide salt with amine-reactive fluorescein derivatives afforded SAENTA-fluorescein conjugates in yields of 50-80%. Chromatographic behavior (Table 1) indicated that the 5-fluorescein conjugates are less polar than the corresponding 6-fluorescein conjugates. In characterization of the SAENTA-fluorescein conjugates, u.v., visible and fluorescence spectral data were used as before[23], and, additionally, ^{1}H NMR and FABMS were used in the structural assignments for all seven SAENTA-fluorescein conjugates. The ^{1}H NMR signal for the fluorescein C-7 hydrogen enabled distinction to be made between

fluorescein 5- and 6-isomers. For the fluorescein C-5 conjugates, this hydrogen resonated at a higher magnetic field, 7-7.4 ppm, (as a doublet, coupling constant 10 Hz) than the corresponding hydrogen in the fluorescein C-6 conjugates, which appeared around 7.7 ppm relative to tetramethylsilane. The spectral data for the SAENTA-fluorescein conjugates showed absorption maxima indicative of the SAENTA and fluorescein moieties in the 262-272 and 496-498 nm regions, respectively.

Although the fluorescence spectral data for Compounds **1-7** (Table 4) confirmed the presence of the fluorescein moiety, both the excitation and emission maxima of the conjugates were red-shifted (1 to 9 nm) relative to those of fluorescein. The fluorescence output of the SAENTA-fluoresceins, which was only 26-39% of that of fluorescein in solution at an excitation wavelength of 488 nm, suggested internal quenching of fluorescein fluorescence in the SAENTA-fluorescein conjugates. The internal quenching was more pronounced in the C-6 conjugates than in the corresponding C-5 conjugates; thus, with each isomeric pair, the more hydrophobic C-5 isomer was also the more fluorescent. Variations in the length and position of attachment of the linker between the SAENTA and fluorescein moieties also changed excitation and emission maxima, as well as fluorescence output. It appeared that the longer the linker, the more intense the fluorescence (Table 4).

***Es* transporter-selective binding.** The recently developed NT mutants of mouse leukemia L1210 cells[25,28] enabled the high affinity of the SAENTA-fluorescein conjugates for the *es* nucleoside transporter to be demonstrated. Binding of the SAENTA-fluorescein conjugate, Compound **4** (6-Sx8-F), to L1210/B23.1 cells imparted fluorescence, whereas cells pretreated with NBMPR, or cells of the clonal line L1210/MA-27.1, which originated from the same clonal line as L1210/B23.1 cells but lack the *es* transporter, were not so stained. Fig. 1 shows the selective nature of the interaction of the conjugate with L1210 cells in histograms of cell-associated fluorescence obtained from flow cytometric analysis. Prior treatment with NBMPR blocked binding of the conjugate to L1210/B23.1 cells (Fig.1b). Thus, the SAENTA-fluorescein conjugate in low concentrations (10 to 20 nM) stained L1210/B23.1 cells (es^+), but not L1210/MA-27.1 cells (es^-) (Fig. 1c). Fig. 1d shows that the fluorescence histograms obtained with L1210/MA-27.1 cells, after staining with Compound **4** in the presence or absence of NBMPR, were superimposable, indicating that, at the particular concentration (15 nM) of Compound **4** used, *es*-selective binding was not detectable in those cells.

The experiment of Fig. 2 showed that the binding of Compound **4** to L1210/B23.1 cells was saturable and *es*-selective in that prior treatment with

TABLE 4. Fluorescence spectra of SAENTA-fluorescein conjugates
Spectra were recorded for conjugates in methanol-buffer solution (methanol added to 10 mM sodium phosphate buffer, pH 7.0 (7:13, v/v)) at 22°C. Uncorrected spectra were obtained as detailed under Analytical Methods. In determination of relative fluorescence output, compounds were excited at 488 nm and emission energy collected through a 530 nm filter was expressed as a percentage of that of fluorescein. The results are presented as means ± S.D. from three separate determinations.

Compound	Excitation λ max (nm)	Emission λ max (nm)	Relative fluorescence output (%)
1*	496	517	27.8 ± 0.6
2	498	519	26.3 ± 0.6
3*	498	521	39.3 ± 2.9
4	498	519	31.3 ± 1.5
5*	498	522	32.0 ± 2.6
6	498	519	29.0 ± 1.7
7*	494	518	32.0 ± 3.5
Fluorescein	493	513	100

* Linkage to the fluorescein C-5 position; other conjugates are linked to C-6.

NBMPR blocked binding of the fluor. These results were typical of the conjugate binding experiments summarized in Table 5. At SAENTA-fluorescein concentrations above 100 nM, binding appeared to be linearly dependent on ligand concentration (data not shown). A similar observation in which binding appeared to be linearly dependent on ligand concentrations beyond 100 nM was attributed to the contribution of fluorescence from free ligand[31].

Mass law analysis of the equilibrium binding data acquired by flow cytometry yielded low K_d values for each of the SAENTA-fluorescein conjugates (Table 5), indicating that the conjugates were tightly bound by the *es* transporter. The low K_i values obtained for the inhibition of site-specific binding of [^{3}H]NBMPR to L1210/B23.1 cells by Compounds **1-4** (Table 5) also indicate that the binding was of high affinity. The tightness of binding of the conjugates for L1210/B23.1 cells correlated with linker length; thus, Compounds **3** and **4** with the 8-atom linker

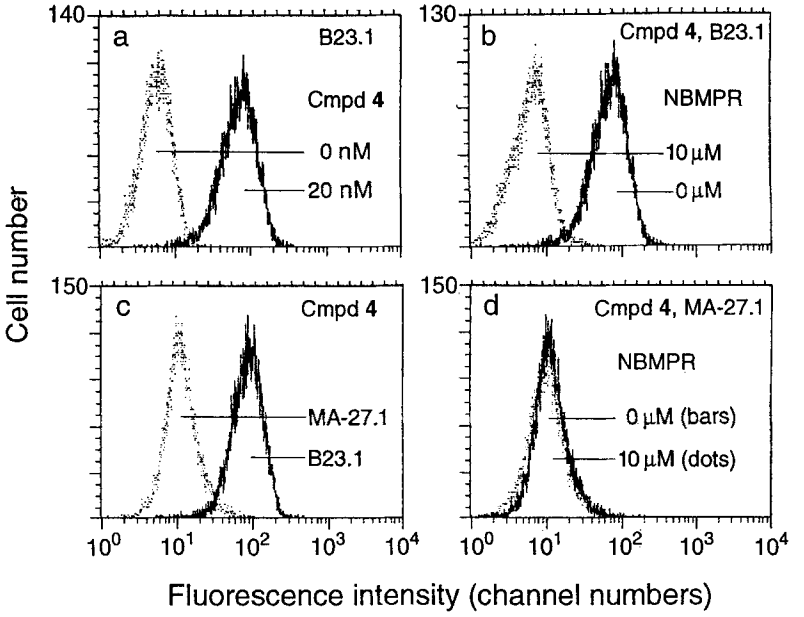


FIG. 1 Binding of 6-Sx8-F (Compound 4) to L1210 cells that express the *es* transporter (L1210/B23.1) or lack the *es* transporter (L1210/MA-27.1). Cells (4×10^5/ml) were incubated with 6-Sx8-F in PBS (pH 8.0) at 22°C for 45 min prior to flow cytometric analysis. Panel a: L1210/B23.1 cells were incubated with (bars) or without (dots) 20 nM 6-Sx8-F, Panel b: L1210/B23.1 cells were incubated with 20 nM 6-Sx8-F in the absence (bars) or presence (dots) of 10 μM NBMPR, Panel c: L1210/B23.1 (bars) or L1210/MA-27.1 (dots) cells were incubated with 15 nM 6-Sx8-F, and Panel d: L1210/MA-27.1 cells were incubated with 15 nM 6-Sx8-F in the absence (bars) or presence (dots) of 10 μM NBMPR.

showed the highest affinity, whereas Compounds **5** and **6** with the shortest linker were among the least tightly bound.

Plots of the ratio $K_d(app)/B_{max}$ *vs* [I], determined in experiments that measured inhibition of [^{3}H]NBMPR binding by the SAENTA-fluoresceins, appeared curved for Compounds **3** and **4** (data not shown), suggesting the presence of more than one type of binding site or binding species. The K_i values reported for Compounds **3** and **4** (Table 5) were obtained by regression analysis of data from the initial linear portions of the curves.

Marked differences were apparent (Table 5) in the maximum cell-bound fluorescence output among the conjugates, as measured by flow cytometry; these differences did not relate to variations in fluorescence output of simple solutions of

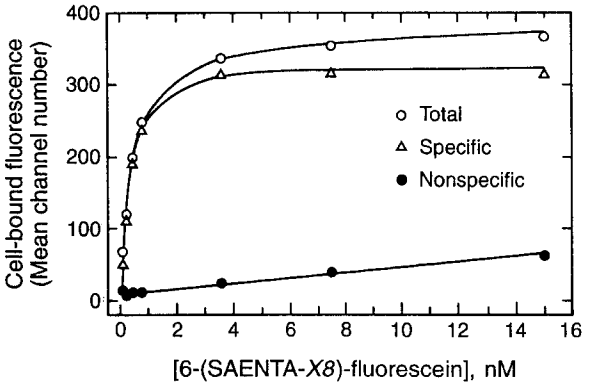


FIG. 2 Flow cytometric analysis of the concentration dependence of *es*-selective binding of 6-Sx8-F (Compound 4) to L1210/B23.1 cells. L1210/B23.1 cells (4×10^5/ml) were incubated with graded concentrations of 6-Sx8-F in PBS (pH 8.0) at 22°C for 45 min, in the absence (○) or presence (●) of 10 μM NBMPR. The specific-binding data (△) represent the difference between the total (○) and nonspecific (●) fluorescence. Medium concentrations of the SAENTA-fluorescein conjugate were determined by fluorimetry at the time of flow cytometric analysis, following centrifugal removal of cells.

TABLE 5. Equilibrium binding of SAENTA-fluorescein conjugates to L1210/B23.1 mouse leukemia cells measured by flow cytometry. K_d values were determined by mass law analysis of equilibrium binding data obtained by flow cytometry. K_i values refer to inhibition of [^{3}H]NBMPR binding. These data were obtained at 22°C and are means ± S.D. from at least three experiments, except in the two experiments with Compound **2**, for which means ± average deviation from the mean are given.

Compound	K_d (nM)	K_i (nM)	B_{max} (mean fluorescence units)
1	26.6 ± 0.4	6.6 ± 1.6	82 ± 45
2	10.9 ± 1.9	12.1 ± 3.8	33 ± 1
3	1.8 ± 0.6	0.95 ± 0.04	25 ± 2
4	4.2 ± 2.8	2.37 ± 0.75	105 ± 27
5	42.9 ± 18.6	-	50 ± 16
6	93.8 ± 44.3	-	60 ± 5
7	2.2 ± 0.7	-	64 ± 8

the SAENTA-fluoresceins (Table 4). As well, relationships were not apparent between maximum cell-bound fluorescence output, binding affinity, or positions of linker attachment.

The *es*-directed binding of the SAENTA-fluorescein conjugates was also demonstrated by the ability of Compound **4** to inhibit [^{3}H]adenosine uptake by L1210/B23.1 cells (data not shown).

DISCUSSION

SAENTA was developed as a tightly-bound probe of the *es* nucleoside transporter that may be coupled to reporter molecules (such as fluorescein) through the primary amino group[26]; that idea led to the development of highly selective fluorescent probes for this transporter (this study). All of the seven conjugates here described were found to be high affinity ligands for the *es* transporter and all served as fluorescent stains for flow cytometry. Lengthening of the spacer arm tended to increase binding affinity (see K_d values, Table 5). Conjugates with linkage attachment to the fluorescein moiety at the C-5 position were somewhat more tightly bound than those with C-6 linkage; however, the number of examples is small and there was no consistent effect of spacer length and/or position of attachment on cell-bound fluorescence output.

The fluorescence output for simple solutions of the conjugates was lower than that for fluorescein (a similar observation was made with Compound **1**[23]), indicating internal quenching, perhaps from interactions between π electrons of the aromatic systems of the SAENTA and fluorescein moieties. The differences in fluorescence yield among the conjugates might result, at least in part, from the conformations conferred by the different linkers and their positions of attachment on the fluorescein molecule. In general, a greater distance between the fluorescein and SAENTA moieties may reduce internal quenching of fluorescence. The influence of conformation on π-π interactions may be the basis of the substantial difference in fluorescence output between Compounds **3** and **4**, which are SAENTA-fluorescein conjugates with the linking groups attached to the C-5 and C-6 positions of fluorescein, respectively; in L1210/B23.1 cells, the fluorescence output from the C-6 conjugate was about 4 times that of the corresponding C-5 conjugate. The 5-(SAENTA)-fluorescein conjugates displayed a larger Stokes shift (the difference between excitation and emission maxima) than did the corresponding 6-fluorescein conjugates.

The high binding affinity of Compound **7** and its high cell-bound fluorescence output are worthy of note. The presence of the triazinyl ring in Compound **7** would

be expected to reduce linkage flexibility relative to the aminocaproyl linkage present in Compounds **3** and **4**, yet Compounds **3**, **4** and **7** have similar binding affinities for L1210/B23.1 cells. The h.p.l.c. R_T value for Compound **7** indicates that it is the most hydrophobic of the seven conjugates. That hydrophobicity and, perhaps, electronic interactions involving the π-electrons of the triazinyl ring, may contribute to the binding of this compound. It is likely that conjugate fluorescence may be influenced by the microenvironment of the binding site. Ganapathi *et al.*[32] observed fluorescence quenching in anthracyclines on binding to cellular macromolecules. The hydrophilicity of the binding site and its vicinity are additional factors that influence ligand fluorescence and binding forces. Stryer[33] has reported that polar binding site environments tended to quench fluorescence, whereas nonpolar environments tended to enhance the fluorescence of 1-anilido-8-naphthalene sulfonate on binding to apoproteins. It may be possible to use changes in the fluorescence of SAENTA-fluorescein conjugates upon binding to probe the microenvironment of the *es* binding site, as has been done for the β-adrenergic receptor binding site[34].

This study showed that SAENTA-fluorescein conjugates with substantial structural differences in linkages between fluorescein and the 5'-carbon of SAENTA were, despite those differences, tightly bound at *es* nucleoside transporter sites on mouse leukemia L1210 cells and on several other lines of cultured neoplastic cells (data not shown). In earlier work, we provided evidence that the *es* sites are accessible at the exofacial aspect of the plasma membrane in erythrocytes[35]. The present results demonstrate that the *es* transporter is remarkably tolerant of SAENTA-conjugated structures distal to the 5'-carbon of SAENTA; we envisage that a variety of reporting groups other than fluorescein may serve in SAENTA conjugates as *es* probes. We note that an earlier report[26] of partial purification of *es* nucleoside transporter polypeptides on a SAENTA-agarose gel matrix also supports the concept of a bulk-tolerant binding site for NBMPR and related ligands in the the *es* transporter.

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