Biochem. J. (1991) 273, 667–672 (Printed in Great Britain)

A new fluorescent probe for the equilibrative inhibitor-sensitive nucleoside transporter

5′-*S*-(2-Aminoethyl)-N^6-(4-nitrobenzyl)-5′-thioadenosine (SAENTA)-x_2-fluorescein

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The N^6-(4-nitrobenzyl) derivative of adenosine is a tight-binding inhibitor of the equilibrative inhibitor-sensitive nucleoside transporter of mammalian cells. A fluorescent ligand for this transporter has been synthesized by allowing an adenosine analogue, 5′-*S*-(2-aminoethyl)-N^6-(4-nitrobenzyl)-5′-thioadenosine (SAENTA), to react with fluorescein isothiocyanate. The purified adduct had a SAENTA/fluorescein molar ratio of 0.92:1 calculated from its absorption spectrum. The intensity of fluorescent emission from the SAENTA-x_2-fluorescein adduct was 30% that of fluorescein isothiocyanate (x_2 is the number of atoms in the linkage between fluorescein and SAENTA). SAENTA-x_2-fluorescein inhibited the influx of nucleosides into cultured leukaemic cells with an IC_{50} (total concentration of inhibitor producing 50% inhibition) of 40 nM. The adduct inhibited the binding of [^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR) with half-maximal inhibition at 50–100 nM. Mass Law analysis of the competitive-binding data suggested the presence of two classes of sites for [^{3}H]NBMPR binding, only one of which was accessible to SAENTA-x_2-fluorescein. Flow cytometry was used to analyse equilibrium binding of SAENTA-x_2-fluorescein to leukaemic cells, and a K_d of 6 nM was obtained. SAENTA-x_2-fluorescein is a high-affinity ligand for the equilibrative inhibitor-sensitive nucleoside transporter which allows rapid assessment of transport capacity by flow cytometry.

INTRODUCTION

In mammalian cells a number of transport processes mediate the entry of purine and pyrimidine nucleosides and of various nucleoside analogues (Paterson *et al.*, 1987; Plagemann *et al.*, 1988; Gati & Paterson, 1989*a*). A major route of nucleoside permeation in erythrocytes and various other types of human cells is equilibrative and highly sensitive to inhibition by S^6-(4-nitrobenzyl) derivatives of 9-β-D-ribofuranosyl-6-thiopurines, such as nitrobenzylthioinosine (NBMPR). By using [^{3}H]NBMPR as a site-specific high-affinity ligand, equilibrative inhibitor-sensitive nucleoside transporters can be enumerated by the technique of equilibrium-binding analysis. Recent studies show that the same transport protein confers specific [^{3}H]NBMPR-binding and transport capacity in erythrocytes and that the stoichiometry of NBMPR binding to the transport site approximates unity (Kwong *et al.*, 1988). Equilibrium binding of [^{3}H]NBMPR has been used to measure the abundance of nucleoside-transport sites in a variety of fresh and cultured human cells (Wiley *et al.*, 1989). However, this assay, which employs a ^{3}H-labelled ligand, cannot identify subpopulations of cells with different transporter densities within a single sample.

A fluorescent ligand for the equilibrative nucleoside transporter would enable ligand–cell interactions to be analysed by flow cytometry, a technique which can differentiate between subpopulations on the basis of light scatter and fluorescence intensity (Sklar, 1987). In addition, flow cytometry requires far fewer cells for rapid analysis of ligand binding. Although a fluorescent dansyl derivative of 6-thioguanosine has been synthesized and shown to bind to the equilibrative nucleoside transporter of human erythrocytes (Shohami & Koren, 1979), this ligand had only moderate affinity (K_d 100–300 nM) as well as high non-specific binding. We have synthesized a more suitable ligand by coupling fluorescein 5-isothiocyanate (FITC) to the amino group of 5′-*S*-(2-aminoethyl)-N^6-(4-nitrobenzyl)-5′-thioadenosine (SAENTA) to produce an analogue that binds tightly to the nucleoside transporter. This fluorescent derivative both inhibits nucleoside transport and competes with NBMPR, a known ligand of the equilibrative nucleoside transporter.

EXPERIMENTAL

Materials

SAENTA phthaloylhydrazide was synthesized as described by Agbanyo *et al.* (1990). FITC Isomer I was obtained from Molecular Probes, Eugene, OR, U.S.A. The silica-gel t.l.c. plates (0.2 mm-thick layers), used to monitor the conjugation of FITC to SAENTA, were from Merck, Darmstadt, Germany. Phthalate oil contained di-n-butyl phthalate (4 vol.) and di-isooctyl phthalate (1 vol.) (BDH, Poole). [^{3}H]NBMPR from Moravek Biochemicals Brea, CA, U.S.A. was purified by reverse-phase h.p.l.c. (Agbanyo *et al.*, 1990). Unlabelled NBMPR was supplied by Sigma (St. Louis, MO, U.S.A.). [^{3}H]Cytosine arabinoside (araC) and [^{14}C]poly(ethylene glycol) were obtained from Amersham, Bucks., U.K. Dilazep was a gift from Roche, Dee Why, Australia.

Abbreviations used: SAENTA 5′-*S*-(2-aminoethyl)-N^6-(4-nitrobenzyl)-5′-thioadenosine; SAENTA-x_2-fluorescein is the fluorescein 5-isothiocyanate adduct of SAENTA, where x_2 is the number of atoms in the linkage between fluorescein and SAENTA; FITC (Isomer 1) fluorescein 5-isothiocyanate; NBMPR, nitrobenzylthioinosine; IBS, imidazole-buffered saline (composition and pH are given in the text); araC, cytosine arabinoside; IC_{50}, total concentration of inhibitor producing 50% inhibition of [^{3}H]cytosine arabinoside influx; R_t, retention time.

Incubations were carried out using imidazole-buffered saline (IBS; 145 mM-NaCl/5 mM-KCl/5 mM-imidazole chloride/1 mM-$MgCl_2$/5 mM-glucose, pH 7.4).

Cell culture

The leukaemic cell line RC2a, of myelomonocytic origin (Bradley *et al.*, 1982) was maintained in RPMI 1640 medium containing 10% (v/v) foetal-calf serum, 2 mM-glutamine, 20 mM-Hepes, pH 7.4 (Flow Laboratories, Sydney, Australia) and 20 mg of gentamycin sulphate/litre (Roussell, Castle Hill, Australia). Cell densities were maintained below 5×10^5/ml.

Synthesis and purification of SAENTA-x_2-fluorescein

SAENTA phthaloylhydrazide was converted into the hydrochloride form by ion-exchange chromatography using AG1-X10 resin (Bio-Rad, Sydney, Australia) with aq. 50% (v/v) methanol as eluant. The synthesis of the SAENTA-x_2-fluorescein adduct was performed by adding equal volumes of SAENTA hydrochloride (1 mM) in aq. 50% methanol to freshly prepared FITC (5 mM) in methanol/0.5 M-sodium bicarbonate buffer, pH 8.5 (1:1, v/v). The reaction was allowed to proceed for 3 h at 37 °C in the dark. Concentrations of SAENTA hydrochloride and FITC were determined spectrophotometrically by using millimolar absorption coefficients for SAENTA hydrochloride (26.9 at 272 nm) and FITC (74 at 495 nm). Progress of the reaction was followed by t.l.c. on silica-gel plates using acetonitrile/aq. 0.1 M-NH_4Cl/aq. 0.1 M-NH_4HCO_3 (6:1:1, by vol.). The t.l.c. plates, examined under u.v. lamps of both long (peak 365 nm) and short (peak 254 nm) wavelengths, clearly distinguished SAENTA(R_F 0.69) from FITC (R_F 0.92). As the reaction progressed, a new spot appeared (R_F 0.84) that absorbed both long- and short-wavelength u.v. light, whereas the SAENTA spot disappeared. When the reaction was complete, the adduct was purified by reverse-phase h.p.l.c. on a C_{18} μBondapak Radial-PAK cartridge (8 mm × 10 cm) (Waters Milford, MA, U.S.A.) using isocratic elution with a methanol/aq. phosphate buffer (10 mM, pH 7.0) (7:13, v/v) and a flow rate of 1 ml/min. The adduct [retention time (R_t) 6.9–8.0 min] was well separated from FITC (R_t 1–4 min). Under these conditions, SAENTA was retained on the column and could only be eluted by lowering the pH of the phosphate buffer component to pH 5.5. Absorption spectra of SAENTA hydrochloride, FITC and SAENTA-x_2-fluorescein were obtained on a DMS200 spectrophotometer (Varian Techtron, Melbourne, Australia). SAENTA-x_2-fluorescein and FITC in phosphate buffer (50 mM, pH 7.4) were examined by fluorescence spectroscopy using a Perkin–Elmer LS-5 spectrometer. The excitation spectra were recorded between 460 and 500 nm, with emission set at 520 nm. The emission spectra were recorded between 490 and 560 nm, with excitation set at 480 nm. Both excitation and emission slits were set at 2.5 nm.

SAENTA-x_2-fluorescein inhibition of [^{3}H]araC influx

Before measurement of [^{3}H]araC uptake, RC2a leukaemic cells were treated at 20 °C for 10 min with graded concentrations of SAENTA-x_2-fluorescein (0–0.25 μM). Treated cells (100 μl; 5×10^7 cells/ml) were added to [^{3}H]araC (100 μl) in IBS medium in 1.5 ml centrifuge tubes to give a final araC concentration of 50 μM. [^{3}H]araC uptake was ended 20 s later by the addition of 800 μl of ice-cold dilazep (750 μM) (Jamieson *et al.*, 1989). Phthalate oil was added immediately and the tube spun at 8000 *g* for 2 min. Medium above the oil was aspirated, the tube walls washed, and the oil above the cell pellet removed. Pellets were solubilized with 0.5 M-NaOH, and the [^{3}H]araC contents were measured by liquid-scintillation counting. The effectiveness of dilazep in stopping influx was confirmed by adding dilazep to cells before the addition of [^{3}H]araC. The uptake of araC was corrected for extracellular [^{3}H]araC trapped under the oil by measuring extracellular space with [^{14}C]poly(ethylene glycol) (5 μCi/ml) in separate tubes.

[^{3}H]NBMPR binding

Washed RC2a cells (1×10^7 cells/ml) were incubated for 30 min at 37 °C with graded concentrations of SAENTA-x_2-fluorescein (0–0.86 μM) and [^{3}H]NBMPR (0.46–8.8 nM total concentration). Determination of the time course for [^{3}H]NBMPR binding (0.46 nM) in the presence of SAENTA-x_2-fluorescein (0.1 μM) established that equilibrium was reached by 30 min. The cells were centrifuged through phthalate oil as described above, and bound [^{3}H]NBMPR was measured by liquid-scintillation counting (Wiley *et al.*, 1982). The free [^{3}H]NBMPR concentration in the medium above the oil was also measured. Non-specific binding of [^{3}H]NBMPR was measured in parallel incubations of cells treated for 5 min at 37 °C with 5 μM-NBMPR before the addition of [^{3}H]NBMPR. Specific binding (total minus non-specific) was used in all analyses.

Analysis of binding data

The results of this competitive binding experiment were analysed by the method of Harris & Winzor (1988), which avoids the presumption that total and free ligand concentrations are identical. Since both ligands (S is [^{3}H]NBMPR and T is SAENTA-x_2-fluorescein) and the transporter protein (A) are presumed to be univalent, eqn. (8) of Harris & Winzor (1988) becomes:

$$(r_t\bar{m}_A/m_S)Z = k_S\bar{m}_A + R(r_t\bar{m}_A/m_S)[k_S(\bar{m}_A - \bar{m}_T) - Zr_t\bar{m}_A/m_S] \quad (1)$$

where m_S and m_T are free concentrations of ligands S and T, $\bar{m}_S$ and $\bar{m}_T$ are corresponding total concentrations of S and T, $\bar{m}_A$ is the total concentration of acceptor (A), k_S and k_T are intrinsic binding constants for ligands S and T, r_t is a binding function defined as fraction of ligand S bound [i.e. $(\bar{m}_s - m_s)/\bar{m}_A$], $R = k_T/k_S$, $Z = 1 + k_S m_S$, and $r_t\bar{m}_A$ is the concentration of bound ligand determined experimentally. The concentration of sites ($\bar{m}_A$) is determined from the abscissa intercept of a Scatchard plot for the binding of [^{3}H]NBMPR in the absence of SAENTA-x_2-fluorescein. Scatchard analysis also provides the value of k_S. A plot of the left-hand side of eqn. (1) versus $(r_t\bar{m}_A/m_S)[k_S(\bar{m}_A - \bar{m}_T) - Zr_t\bar{m}_A/m_S]$ is linear with slope $R(= k_T/k_S)$ and obligatory ordinate intercept $k_S\bar{m}_A$ when the inhibition is simple competitive in type.

Flow-cytometric binding studies

Cell-bound fluorescence was measured on an EPICS V 741 flow cytometer (Coulter, Hialeah, FL, U.S.A.) fitted with an argon laser. Excitation was at 488 nm and the fluorescence emission was collected between 515 and 530 nm.

Forward-angle and right-angle scatter signals were also collected and used to eliminate fluorescent signals associated with cell debris. SAENTA-x_2-fluorescein binding studies with RC2a leukaemic cells (10^5 cells/ml in phosphate-buffered saline) were carried out at room temperature. Histograms of relative intensities of cell-associated fluorescence signals were collected under the following conditions: (*a*) before any addition of ligand (autofluorescence), (*b*) after addition of 0–100 nM-SAENTA-x_2-fluorescein and (*c*) after a further addition of 1 μM-NBMPR or 2 μM-dilazep. The mean fluorescence intensity was calculated using integrated signals (i.e. the integral of fluorescence intensity over time taken for each cell to pass through the laser beam) from a minimum of 2500 cells. This technique allowed relative fluorescence intensities to be monitored continuously against time to ensure that equilibrium between bound and free

SAENTA-x_2-fluorescein was reached under each condition. At the lowest ligand concentration equilibrium was reached after 10 min.

RESULTS

Characterization of SAENTA-x_2-fluorescein

The absorption spectrum of the adduct in methanol/aq. phosphate buffer (10 mM, pH 7.0) (7:13, v/v) was similar to the spectrum of FITC in the region of 350–600 nm, but showed an increased absorption below 350 nm, consistent with a contribution by SAENTA (see Fig. 1). The ratio of absorbances at 272 nm and 495 nm of 0.32 ± 0.015 was established for FITC ($n = 28$). By using this ratio and the absorption coefficients for SAENTA and FITC, a molar ratio of 0.92:1 for SAENTA/fluorescein was calculated for the adduct. This result is consistent with the structure of the adduct shown in Fig. 2. The fluorescence excitation and emission spectra of the adduct were compared with those obtained for an equimolar solution of FITC in aq. phosphate buffer, pH 7.4 (Fig. 3). The intensity of fluorescence emission from SAENTA-x_2-fluorescein adduct was 30% that of FITC, and the emission spectrum showed a maximum at 514 nm which was blue-shifted by 3 nm relative to the FITC spectrum (Fig. 3).

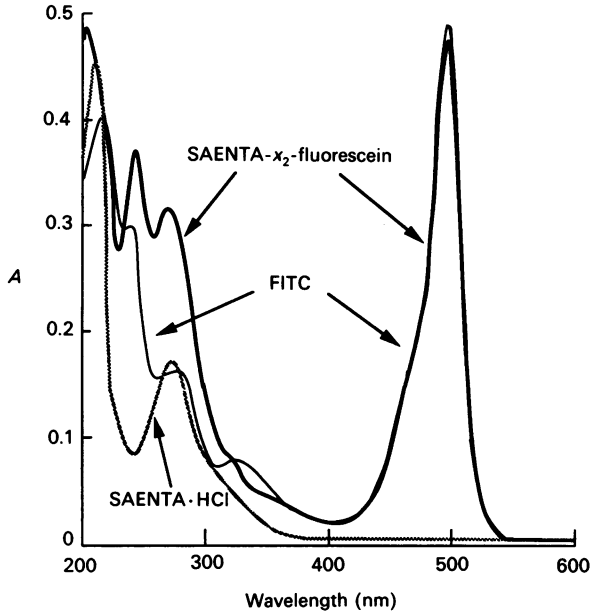

Fig. 1. Absorption spectra of FITC, SAENTA hydrochloride (·HCl) and SAENTA-x_2-fluorescein in methanol/aq. 10 mM-phosphate buffer, pH 7.0 (7:13, v/v)

The concentration of the SAENTA-x_2-fluorescein was adjusted so that the absorption at 495 nm was equal to that obtained with the FITC solution. All spectra were corrected for the solvent baseline. The spectrum of SAENTA-x_2-fluorescein showed a maximum at 495 nm, characteristic of a fluorescein derivative, as well as a maximum at 268 nm, consistent with a contribution by SAENTA.

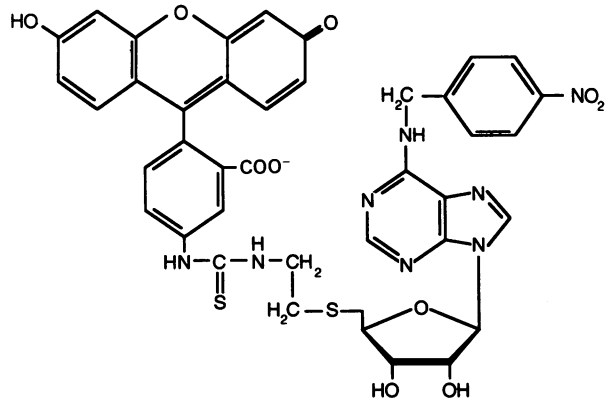

Fig. 2. Structure of SAENTA-x_2-fluorescein

Inhibition of nucleoside transport

The ability of SAENTA-x_2-fluorescein to inhibit influx of nucleoside was assessed in cultured RC2a leukaemic cells. Influx of [^{3}H]araC (50 μM) at 20 °C was inhibited by 90% after exposure of cells for 10 min to 0.25 μM-SAENTA-x_2-fluorescein (Fig. 4). The IC_{50} for the SAENTA-x_2-fluorescein was estimated to be 40 nM.

Inhibition of [^{3}H]NBMPR binding

The binding of [^{3}H]NBMPR to cultured RC2a leukaemic cells was measured in the presence or absence of unlabelled NBMPR (5 μM). The difference represents specific binding, which, in this

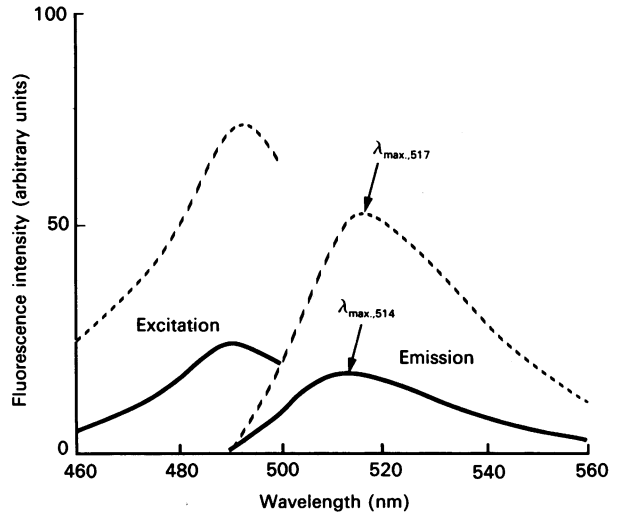

Fig. 3. Excitation and emission spectra of SAENTA-x_2-fluorescein (——) and FITC (– – – –) in 50 mM-phosphate buffer, pH 7.4

A solution of SAENTA-x_2-fluorescein was diluted so that its A_{495} was equal to that of 1.5 μM-FITC. The excitation spectra of both solutions were obtained with a fixed emission wavelength of 520 nm, and an excitation of 480 nm was used for the emission spectra.

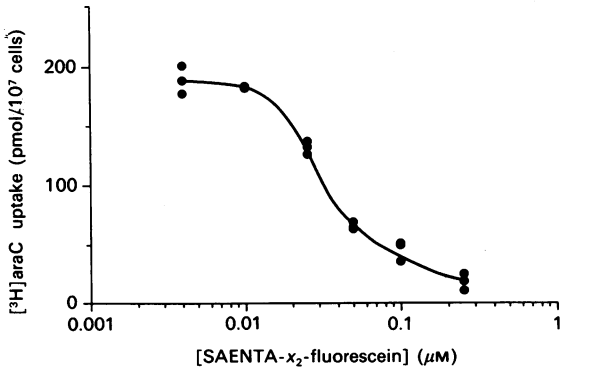

Fig. 4. Inhibition of araC influx by increasing concentrations of SAENTA-x_2-fluorescein

RC2a leukaemic cells were treated for 10 min with graded concentrations of SAENTA-x_2-fluorescein (0–0.25 μM). Rates of uptake of araC (50 μM) were then determined over 20 s at 20 °C.

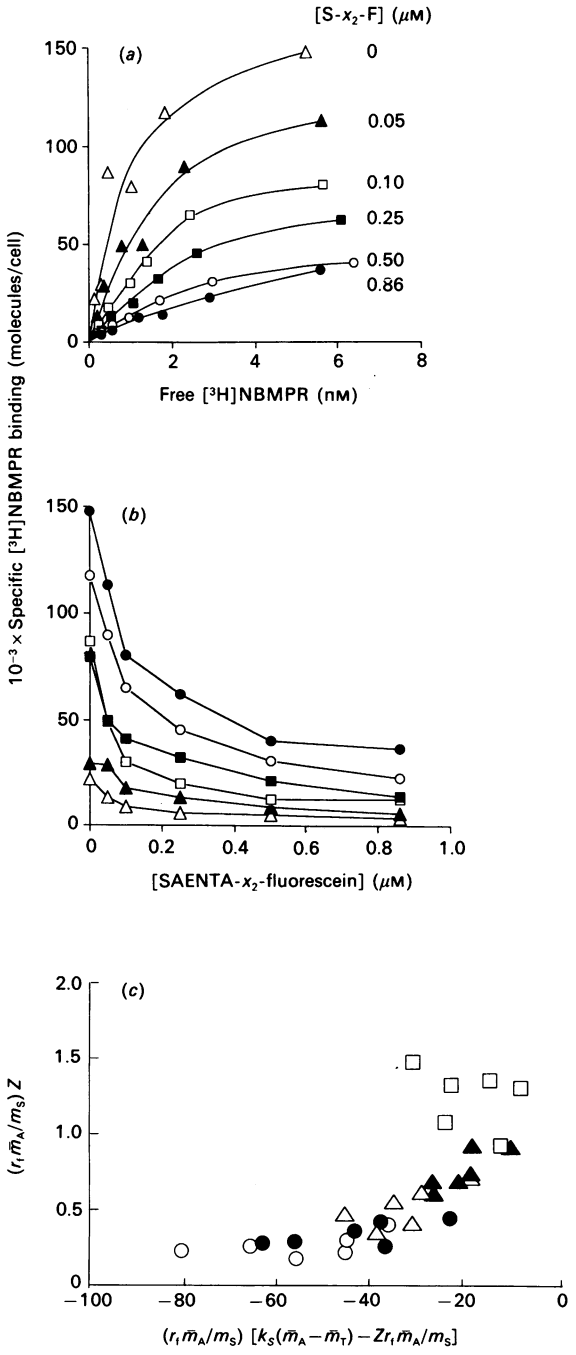

Fig. 5. Inhibition of [^{3}H]NBMPR binding by increasing concentrations of SAENTA-x_2-fluorescein

(*a*) Inhibition of specific [^{3}H]NBMPR binding by SAENTA-x_2-fluorescein (S-x_2-F). Various concentrations of [^{3}H]NBMPR were added simultaneously with SAENTA-x_2-fluorescein (0–0.86 μM) to suspensions of RC2a leukaemic cells, which were incubated for 30 min at 37 °C. Specific [^{3}H]NBMPR binding was calculated by subtracting the amount of non-specific binding (determined in the presence of 5 μM unlabelled NBMPR) from total [^{3}H]NBMPR binding. (*b*) Specific [^{3}H]NBMPR binding is not entirely inhibited by increasing concentrations of SAENTA-x_2-fluorescein. The data of (*a*) were plotted to show the effect of SAENTA-x_2-fluorescein on specific binding of [^{3}H]NBMPR at total concentrations of 8.77 nM (●), 4.41 nM (○), 2.69 nM (■), 1.81 nM (□), 0.91 nM (▲) and 0.46 nM (△). (*c*) Analysis of SAENTA-x_2-fluorescein inhibition of specific [^{3}H]NBMPR binding in RC2a cells, according to eqn. (8) of Harris & Winzor (1988). The data from (*a*) were plotted for SAENTA-x_2-fluorescein concentrations of 0.05 μM (□), 0.10 μM (▲), 0.25 μM (△), 0.50 μM (●) and 0.86 μM (○).

cell line, constitutes a high proportion (82–96%) of the total binding of this ligand. The fluorescent adduct SAENTA-x_2-fluorescein (0–0.86 μM) produced a concentration-dependent inhibition of specific [^{3}H]NBMPR binding, with half-maximal inhibition observed at 50–100 nM (Fig. 5*a*). The data were replotted (Fig. 5*b*) to illustrate the component of specific [^{3}H]NBMPR binding that was not displaced by even the highest concentration of SAENTA-x_2-fluorescein (0.86 μM). That the fluorescent ligand does not compete for all the binding sites available to [^{3}H]NBMPR is re-emphasized in Fig. 5(*c*), in which the results have been subjected to analysis in terms of competitive binding between two ligands for the same sites (eqn. 1). The curvilinear form of this experimental plot has been predicted (Harris & Winzor, 1988) for situations in which the second ligand (T) only competes for a fraction of the binding sites available to the first ligand (S).

Equilibrium binding of SAENTA-x_2-fluorescein measured by flow cytometry

RC2a cells were incubated with a range of SAENTA-x_2-fluorescein concentrations (0–100 nM) at room temperature and the cell-associated fluorescence analysed by flow cytometry. The intensity of cell-bound fluorescence increased and reached equilibrium values after 10 min of incubation at all ligand concentrations. The histograms of cell-bound fluorescence obtained at equilibrium (Fig. 6) show that addition of 1 μM-NBMPR reduced the binding of SAENTA-x_2-fluorescein. This NBMPR-sensitive ligand binding was measured at each SAENTA-x_2--fluorescein concentration and plotted against the initial ligand concentration (Fig. 7). Low cell concentrations (1×10^5/ml) were employed in these experiments to minimize depletion of SAENTA-x_2-fluorescein from the medium. Under these conditions the concentration of SAENTA-x_2-fluorescein giving half-maximal binding was 6.2 ± 1.0 nM ($n = 3$, ± 1 S.D.) (Fig. 7), a value which should approximate the K_d for specific binding (Sklar, 1987). The specificity of SAENTA-x_2-fluorescein binding was further confirmed by measurements in the presence of dilazep, a potent inhibitor of nucleoside transport structurally unrelated to NBMPR (Gati & Paterson, 1989*b*). Dilazep (2 μM) was as effective as NBMPR (1 μM) in displacing SAENTA-x_2-fluorescein binding (results not shown).

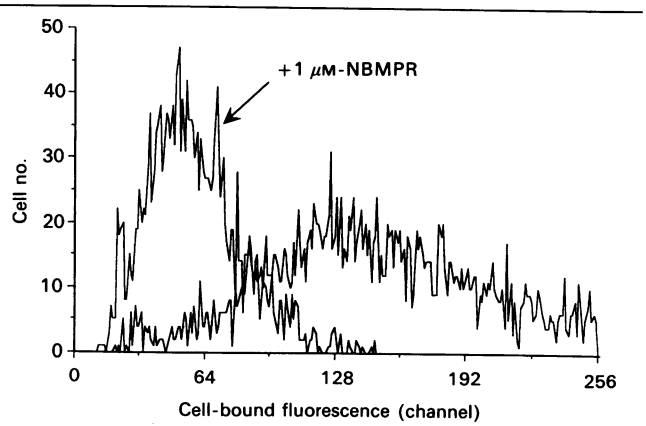

Fig. 6. Flow-cytometric analysis of SAENTA-x_2-fluorescein binding and its inhibition by NBMPR

RC2a leukaemic cells were incubated with 100 nM-SAENTA-x_2-fluorescein for 10 min at room temperature. NBMPR (1 μM) was then added and the cells incubated for a further 10 min. Histograms of cell-bound fluorescence were obtained before and after addition of NBMPR by flow-cytometric analysis of approx. 2500 cells. The integrated fluorescence signals generated from individual cells were assigned into a 256-channel trace on the basis of increasing fluorescence intensity.

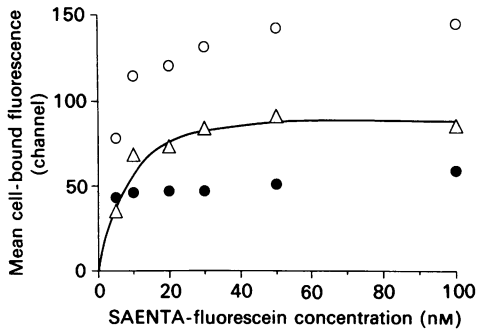

Fig. 7. Equilibrium binding of SAENTA-x_2-fluorescein measured by flow cytometry

RC2a leukaemic cells (10^5 cells/ml) were incubated for 10 min at room temperature with a range of SAENTA-x_2-fluorescein concentrations (0–100 nM) in the absence (○) or presence (●) of 1 μM-NBMPR. The difference between these two signals represents NBMPR-sensitive SAENTA-x_2-fluorescein binding (△). Cell-bound fluorescence has not been corrected for autofluorescence, which contributes 20–25 channels.

DISCUSSION

This paper describes the synthesis and properties of a fluorescent probe of the equilibrative nucleoside transporter. The probe is an adduct between fluorescein and a derivative of nitrobenzyladenosine, a tight-binding inhibitor of nucleoside transport (Paterson *et al.*, 1983). The proposed chemical structure of the SAENTA-x_2-fluorescein adduct (Fig. 2) is consistent with the absorption spectrum (Fig. 1) which approximates the sum of the individual spectra of SAENTA and fluorescein. Fluorescence emission intensity of SAENTA-x_2-fluorescein was only 30% of the intensity of FITC (Fig. 3). Assuming identical fluorescein absorption coefficients for both compounds, it can be concluded that the proximity of the 6-nitrobenzyl group or the purine moiety to the fluorophore in SAENTA-x_2-fluorescein quenches its fluorescence. S^6-(4-nitrobenzyl) derivatives of 6-thiopurine nucleosides (e.g. NBMPR) and N^6-(4-nitrobenzyl) derivatives of adenosine (e.g. acetyl-SAENTA) are well-characterized tight-binding inhibitors of mammalian nucleoside transport (Paterson *et al.*, 1983, 1987; Agbanyo *et al.*, 1990). This study shows that the SAENTA-x_2-fluorescein adduct is also a strong inhibitor of nucleoside transport. Influx of [^{3}H]araC into cultured leukaemic cells was inhibited with an IC_{50} of 40 nM (Fig. 4) for the adduct, compared with an IC_{50} of 6 nM for NBMPR (Jamieson *et al.*, 1989). Since the only significant route of entry of araC in RC2a cells is by the equilibrative inhibitor-sensitive pathway (Jamieson *et al.*, 1989), it can be concluded that SAENTA-x_2-fluorescein is inhibiting this system. Moreover, SAENTA-x_2-fluorescein inhibited the binding of [^{3}H]NBMPR to leukaemic cells in a concentration-dependent manner (Fig. 5).

Because flow-cytometric measurements showed that binding of SAENTA-x_2-fluorescein to RC2a cells was inhibited by NBMPR (Fig. 6) these ligands appear to interact with a common site. However an analysis of the data did not conform to simple competitive binding, and led to a more rigorous analysis (Figs. 5*b* and 5*c*) as described by Harris & Winzor (1988). The non-linearity of Fig. 5(*c*) is of interest in that it suggests the existence of two classes of NBMPR-binding sites that have similar affinity for NBMPR, but only one of which can interact with SAENTA-x_2-fluorescein. The nature of the sites that are accessible to [^{3}H]NBMPR but not to SAENTA-x_2-fluorescein is uncertain. However, NBMPR may be more lipid-soluble than SAENTA-x_2-fluorescein and might bind to newly synthesized transporter molecules contained in an intracellular location.

Flow cytometry enables the amount of cell-bound fluorescent ligand to be directly measured under equilibrium conditions (Sklar & Finney, 1982; Chatelier *et al.*, 1986; Sklar, 1987). Most of the fluorescent signal arising from SAENTA-x_2-fluorescein bound to human cultured leukaemic cells is displaced by subsequent addition of 1 μM-NBMPR. This NBMPR-sensitive component of SAENTA-x_2-fluorescein binding is saturable with increasing concentrations of SAENTA-x_2-fluorescein. Flow-cytometric analysis makes it possible to use cell concentrations as low as 10^5 cells/ml in binding studies, allowing less than 1% of the total ligand to be bound, so that the free ligand concentration closely approximates the total ligand concentration added. Making this assumption, the K_d for the binding of SAENTA-x_2-fluorescein to leukaemic RC2a cells is 6 nM. This value, which is higher than the K_d of NBMPR binding (0.3–1 nM) to erythrocytes or fresh leukaemic cells (Jarvis *et al.*, 1982; Wiley *et al.*, 1982), is lower than that obtained for the binding of acetyl derivatives of SAENTA (76 nM) (Agbanyo *et al.*, 1990). SAENTA-x_2-fluorescein has significant advantages over another fluorescent ligand, dansylaminoethyl-6-thioguanosine, which was synthesized and used as a probe for the nucleoside-transport system in human erythrocytes (Shohami & Koren, 1979). However, dansyl derivatives require excitation in the u.v. (at 310 nm) making them less suitable than SAENTA-fluorescein as ligands for flow cytometry. Dansyl derivatives have been used as environment-sensitive fluorescent probes, since large changes in emission wavelength, quantum yield and lifetime result from changes in the polarity around the dansyl group. By contrast, fluorescein derivatives are more suitable for quantitative studies, since fluorescent emission is less influenced by the polarity of its microenvironment and have a higher quantum yield than has the dansyl fluorophore (Pesce *et al.*, 1971).

The success of SAENTA-x_2-fluorescein as a fluorescent ligand for the nucleoside transporter is a consequence of fluorescein being conjugated to the 5′-position of the ribose in the nucleoside, a site on the ligand which tolerates bulky substituents without interfering with the binding reaction of the nucleoside. It is known that bulky substituents on the 2′ or 3′ position of the ribose ring interfere with interaction of nucleosides with the transporter (Gati *et al.*, 1983, 1984), while S^6-eosin derivatives of 6-thioguanosine do not affect either [^{3}H]NBMPR binding or nucleoside transport in RC2a leukaemic cells (G. P. Jamieson & M. B. Snook, unpublished work). SAENTA-x_2-fluorescein holds promise as the prototype of a class of fluorescent or phosphorescent SAENTA-based ligands for the nucleoside transporter.

We thank Dr. D. Winzor for helpful discussion. We thank Elaine Brigal for typing the manuscript. This work was supported by the Anti-Cancer Council of Victoria, Australia, the Medical Research Council of Canada, the National Cancer Institute of Canada, the Alberta Cancer Board Research Initiatives Program, and the Natural Sciences and Engineering Council of Canada. C. E. C. is a Senior Research Scientist of the National Cancer Institute of Canada.

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Received 9 April 1990/21 August 1990; accepted 28 August 1990