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Use of a Small Reporter Molecule To Determine Cell-Surface Proteins by Capillary Electrophoresis and Laser-Induced Fluorescence: Use of 5-SAENTA-x8f for Quantitation of the Human Equilibrative Nucleoside Transporter 1 Protein

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The human equilibrative nucleoside transporter 1 protein (hENT1) is a major mediator of cellular entry of nucleosides and anticancer nucleoside drugs; its assay is important in understanding and diagnosing chemotherapy resistance. Here we present a novel assay for quantifying hENT1 using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). A cellular population is treated with 5′-*S*-(2-aminoethyl)-*N*^{6}-(4-nitrobenzyl)-5′-thioadenosine-x8-fluorescein (5-SAENTA-x8f), which binds with high affinity and specificity to the hENT1 protein. The cells are washed to remove excess reagent, lysed, and centrifuged, and the supernatant is analyzed by CE-LIF with the use of an internal standard. Accuracy was evaluated by comparing the capillary electrophoresis results with those obtained by flow cytometry; the results were highly correlated, $r = 0.96$. The relative standard deviation of the hENT1 assay was 10%, determined from nine independent assays of the same cell line, which is 3 times superior to results obtained in a flow cytometry assay. The detection limit for 5-SAENTA-x8f was 4300 molecules injected into the capillary.

A typical eukaryotic cell contains perhaps 10 000 proteins. A fraction of these proteins are membrane-bound receptor and transporter molecules, which are often targets for pharmacology and diagnosis. The assay of a specific cell-surface protein requires either the use of an exhaustive separation method to isolate the protein or the use of affinity interaction with a specific reporter molecule. The former method is unacceptably cumbersome except for the most highly expressed proteins. The latter method relies on the use of a labeled affinity probe, which most often is an antibody raised against the target protein.[1−2] However, antibodies are difficult to generate, suffer from nonspecific binding to related molecules, and do not discriminate between active and inactive forms of the target protein. It is instead preferable to use a small molecule as a specific affinity probe to minimize nonspecific binding and to target functional proteins. In this paper, we demonstrate and validate the use of a fluorescent probe molecule with capillary electrophoresis and laser-induced fluorescence for the assay of the human equilibrative nucleoside transport protein (hENT1), which is a transporter protein that is implicated in resistance to anticancer nucleosides.

Cytotoxic chemotherapy is the mainstay of the clinical management of systemic cancers.[3,4] Among the several classes of antitumor agents, cytotoxic nucleoside drugs are potent antimetabolites that display antitumor activities against both hematologic malignancies and solid tumors.[5] The nucleoside anticancer drugs in routine clinical use are fludarabine (9-β-D-arabinosyl-2-fluoroadenine), cladribine (2-chloro-2′-deoxyadenosine), cytarabine (1-β-D-arabinofuranosylcytosine), and gemcitabine (2′,2′-difluorodeoxycytidine).[6] Each agent is a hydrophilic compound that requires cellular uptake by functional plasma membrane nucleoside transport (NT) proteins to permeate the plasma membrane and to interact with its intracellular targets.[7−10]

Five distinct human NT proteins, varying in substrate specificity, sodium ion dependence, and sensitivity to inhibition by nitrobenzylthioinosine (NBMPR, Figure 1) have been identified by molecular cloning and functional expression of cDNAs from human tissues.[11] One transporter, the hENT1 protein, appears to be ubiquitous in human cells, has functional characteristics of *es-*

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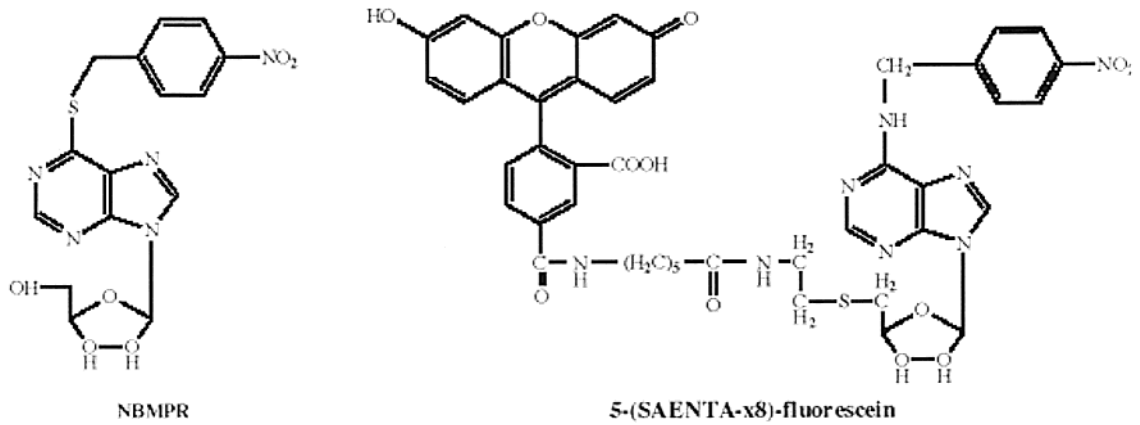

Figure 1. Structure of hENT1 inhibitors. 5-SAENTA-x8f is a modified form of nitrobenzyladenosine to which is attached a fluorescein molecule.

mediated transport (*e*quilibrative and sensitive to inhibition by nanomolar concentrations of NBMPR), and mediates cellular entry of many cytotoxic nucleosides, including the four clinically used drugs described above.[10−12]

Unfortunately, a large percentage of tumors are resistant to treatment by the cytotoxic nucleosides; patients with drug-resistant tumors gain no benefit from treatment but suffer significant immunosuppression and hematologic toxicity.[13] Because hENT1 appears to be the major mediator of nucleoside drug influx in normal and malignant human blood cells, it has been suggested that hENT1 deficiency is likely the mechanism of transport-based nucleoside drug resistance.[7,14] Its determination is therefore important in understanding the details of and in developing assays for chemotherapy resistance.

hENT1 is a 456-residue protein of M_r 50 249 with 11 transmembrane segments (N terminus intracellular and C terminus extracellular) and three potential N-glycosylation sites.[15] The number of hENT1 sites on cells has traditionally been estimated by steady-state binding of [^{3}H]NBMPR, a high-affinity (K_d values, 0.1−1 nM) specific inhibitor of es-mediated transport of nucleosides in mammalian erythrocytes and a variety of other cell types.[16,17] However, this assay, which involves radiolabeled reagents, requires large numbers of cells (in excess of 10^8) because of the relatively small number (10^4−10^6) of inhibitor binding sites per cell.[18]

Recent studies that demonstrated tolerance for introduction of substituents at the 5′-position of the ribosyl moiety of es transport inhibitors led to the development of 5′-*S*-(2-aminoethyl)-*N*^{6}-(4-nitrobenzyl)-5′-thioadenosine (SAENTA), a derivatizable analogue of NBMPR.[19] Substituents at the 5′-position of NBMPR were shown to have little effect on the potency of NBMPR as an inhibitor of nucleoside transport activity.[20] Conjugates of SAENTA in which the nucleoside analogue is linked to fluorescein have proven to be specific stains for cellular NBMPR binding sites.[21−24] Thus, the SAENTA-fluoresceins allow measurement of the relative abundance of NBMPR-binding sites of cells by a procedure that requires 10^7 cells, which is 10% of the cells needed for assays with radiolabeled ligands.[24−25] Despite the use of flow cytometry to quantify NBMPR-binding sites, the importance of deficiencies in nucleoside transport capacity in clinical drug resistance remains unclear, in large part due to the problems associated with quantifying nucleoside transport proteins in malignant clones admixed with normal cells[11] and the inability of flow cytometry to demonstrate subpopulations of transporter-deficient cells. Current assays cannot be applied to solid tumors because of the technical difficulties in separating large numbers of malignant cells from contaminating benign stromal cells. For these reasons, we sought a new approach for hENT1 quantitation that has the potential to quantitate the hENT1 protein abundance in single cells.

In this work, we demonstrate hENT1 quantitation using CE followed by laser-induced fluorescence (LIF) detection based on 5-SAENTA-x8-fluorescein (5-SAENTA-x8f, structure shown in Figure 1), which binds with high affinity and specificity to hENT1 in a 1:1 stoichiometry.[26,27] Results obtained with the hENT1 CE-LIF assay are similar to those obtained by flow cytometry. The

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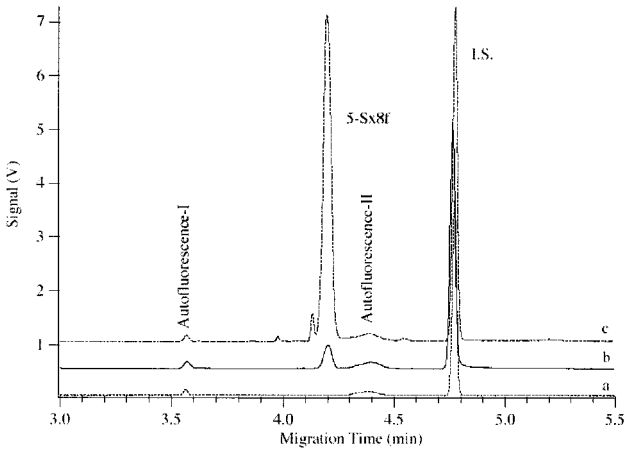

Figure 2. Detection of 5-SAENTA-x8f released from the binding complex from 435S cells. (a). Negative control sample in which no 5-SAENTA-x8f was added in the initial incubation mixture. No 5-SAENTA-x8f signal was observed in this case. (b). CE sample prepared as described in the Experimental Section in which a certain amount of 5-SAENTA-x8f was added in the initial incubation mixture. (c) Sample b was spiked with 5-SAENTA-x8f standard to confirm the identification of 5-SAENTA-x8f.

instrument detection limits suggest the potential of the hENT1 CE-LIF assay for single-cell quantitation of NBMPR-binding sites. Further refinement of this technique might thereby permit assays of clinical samples to (i) quantify hENT1 on malignant cells admixed with cells and and (ii) identify hENT1-deficient (and thereby nucleoside drug-resistant) subclones among malignant cells. This information could then be used to validate the relationship between hENT1 quantity and clinical responsiveness in nucleoside-treated cancers and, potentially, to choose optimal candidates for nucleoside anticancer therapy.

EXPERIMENTAL SECTION

Reagents. 5-SAENTA-x8f was a generous gift from James Wiley of Sydney University, Penrith, Australia. Other reagents were from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

Buffers and Solutions. The following buffers were used. (i) 5-SAENTA-x8f solutions were prepared with a buffer containing 10 mM HEPES, 100 mM NaCl, and 5 mM KCl at pH 7.3; (ii) cells were suspended and washed with 10 mM HEPES, 100 mM NaCl, 5 mM KCl, 1 g/L glucose, and 1 g/L BSA at pH 7.3; (iii) 5-SAENTA-x8f bound to nonspecific sites on the cell other than hENT1 sites was removed by washing the cells with a nonionic surfactant, 0.1% (v/w) *n*-octyl glucoside with 1 g/L glucose and 1 g/L BSA; (iv) CE electrophoretic buffer consisted of 10 mM Borate and 5 mM sodium dodecyl sulfate at pH 8.9. Solutions were filtered through a 0.22-μm membrane filter (Millipore, Bedford, MA) before use.

Cell Culture. Two human breast carcinoma cell lines (MCF-7 and MDA-MB-435s), a human colon carcinoma cell line (CaCo-2), and a human cervical carcinoma cell line (HeLa) were obtained from the American Type Culture Collection. CCI-180/1 cells were established from a biopsy of a stage Ib uterine squamous cell cervical cancer. HeLa cells were grown in Roswell Park Memorial Institute (RPMI) supplemented with 10% fetal calf serum, and the other four cell lines were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum; all cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air and were free of mycoplasma. Cell concentrations were determined using a hemocytometer. Cultures were removed from dishes with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA·4Na) and subcultured every 3−4 days. Experiments were initiated with cells in the exponential growth phase.

hENT1 Quantitation Assay. Cell suspensions (5×10^5) were added to graded concentrations of 5-SAENTA-x8f (0−50 nM) solutions. 5-SAENTA-x8f is a nonpermeating reagent because of its size and charge and only binds to external NBMPR-binding sites. 5-SAENTA-x8f was incubated with cells for 50 min at room temperature. Incubations were terminated by centrifugation at 150*g* for 10 min. (All centrifugations were done under this condition unless stated otherwise.) The pelleted cells were washed twice with buffer ii to remove the extra 5-SAENTA-x8f and once with surfactant iii to remove nonspecifically bound 5-SAENTA-x8f.[19] Cells were then centrifugally pelleted and lysed with CE buffer, releasing 5-SAENTA-x8f from the binding complex. The solutions were centrifuged at 9300*g* for 10 min, and the supernatants were sampled for CE measurement of 5-SAENTA-x8f binding content. Calibration curves were constructed using a serial dilution of a 5-SAENTA-x8f standard for quantitation. Three additional samples (controls), which were washed to the same extent but without being lysed, were used to obtain cell numbers.

Instrumentation and Capillary Electrophoretic Conditions. A locally constructed CE-LIF instrument was used for our study and is described in detail elsewhere.[28,29] Each sample was mixed with an injection standard of fluorescein and introduced electrokinetically into the capillary. A 30-cm-long, 50-μm-inner diameter, 150-μm-outer diameter uncoated fused-silica capillary (J&W Scientific, Folsom, CA) was used for the separation. The electrophoretic buffer was renewed every five or six runs. All experiments were carried out by applying a constant voltage of 9 kV. Fluorescence from 5-SAENTA-x8f was excited with an argon ion laser (Melles Griot Laser Group, Carlsbad, CA) operating at 488 nm. Emission was collected through a 535-nm band-pass filter of 35-nm width (535DF35, Omega Optical, Brattleboro, VI) and detected by a R1477 photomultiplier tube. Data were collected using software written in Labview and analyzed using Igor Pro 2.04 (WaveMetrics, Lake Oswego, OR) and Matlab (Mathworks, Natick, MA). In all experiments, the 5-SAENTA-x8f signal was divided by the signal of the injection standard to produce a normalized signal.

Flow Cytometry. 5-SAENTA-x8f was used for flow cytometric enumeration of transport sites on cultured human epithelial malignant cells maintained as described above.[20,30,31] Trypsinized cells were washed and suspended in flow buffer (140 mM NaCl, 5 mM KCl, 10 mM HEPES pH 7.4, 1 g/L glucose, and 1 g/L

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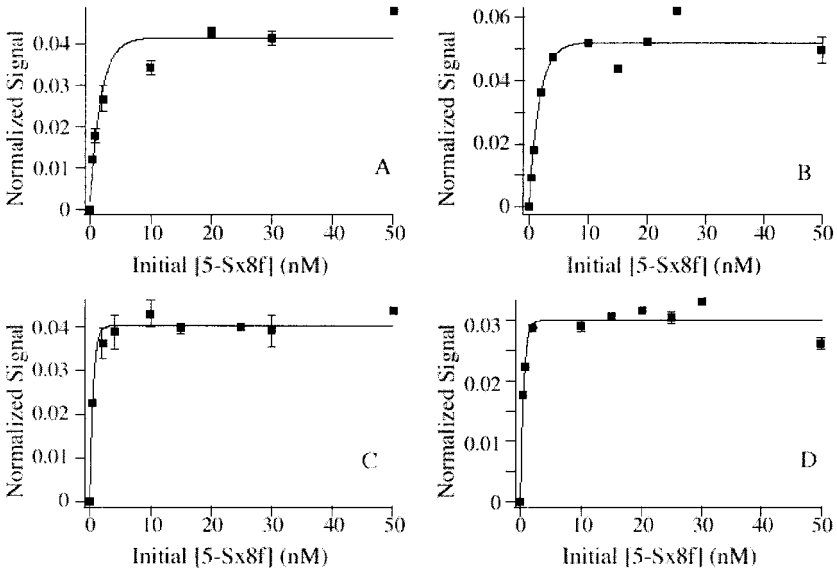

Figure 3. Equilibrium binding analysis of 5-SAENTA-x8f as a surrogate marker for the presence of the hENT1 protein: (A) 2.5×10^5 435S cells, $K_d = 1.30$ nM. (B) 2.3×10^5 435S cells, $K_d = 1.33$ nM. (C) 2.4×10^5 MCF-7 cells, $K_d = 0.33$ nM. (D) 1.5×10^5 MCF-7 cells, $K_d = 0.28$ nM. All data points were the average of two independent measurements, and the error bar shows the standard deviation.

Table 1. Number of Binding Sites of Five Cultured Human Cancer Cell Lines

	CE-LIF		flow cytometry	
cell lines	hENT1 binding sites/cell	RSD[a]	hENT1 binding sites/cell	RSD[a]
435S	2.0×10^5	20	1.7×10^5	24
HeLa	1.5×10^5	6	1.1×10^5	41
MCF-7	1.3×10^5	23	1.5×10^5	44
CaCo-2	2.0×10^5	17	1.7×10^5	54
180/1	2.4×10^5	16	2.2×10^5	14

[a] The relative standard deviation (RSD, %) is calculated based on at least three independent experiments.

Table 2. K_d Values for hENT1 Binding in Five Cultured Human Cell Lines

cell line	K_d (nM)
435S	1.32
HeLa	0.92
MCF-7	0.31
CaCo-2	0.94
180/1	0.86

bovine serum albumin). A total of 5×10^5 cells were then incubated for 10 min at room temperature in the presence or absence of 1 mM NBMPR. Samples were than incubated in graded concentrations of 5-(SAENTA-x8f)-fluorescein (final concentration of 1−20 nM) at room temperature for 30 min. Cell-bound fluorescence and light scatter signals for 10 000 cells were analyzed on a FACSort instrument (Becton Dickinson Canada, Oakville, ON, Canada). Calibration curves were constructed using fluorescein standard beads containing known numbers of fluorescein molecules. Mean fluorescence channel numbers were converted to molecules of equivalent soluble fluorescein (MESF; Flow Cytometry Standards Corp., Research Triangle Park, NC). The specific values for bound fluorescence for each sample were divided by 0.41, the previously validated MESF/NBMPR binding site ratio, to obtain plasma membrane hENT1 sites per cell.[32]

RESULTS AND DISCUSSION

Capillary electrophoresis was used to detect 5-SAENTA-x8f. A linear calibration curve ($r = 0.998$, $n = 8$) was obtained with 5-SAENTA-x8f concentrations ranging from 1×10^{-10}−2×10^{-8} mol/L. Nine aliquots of the same sample were analyzed; the relative precision in migration time was 0.7% and in peak height was 6.7%. The instrumental limit of detection (3σ) was 1600 molecules for fluorescein and 4300 molecules for 5-SAENTA-x8f.[33] The results are consistent with the previous observation that fluorescent output for 5-SAENTA-x8f is ∼39% of fluorescein.[34] 5-SAENTA-x8f generates a relatively broad peak compared with fluorescein; this increased peak width is likely due to the presence of geometrical isomers of 5-SAENTA-x8f.

5-SAENTA-x8f was then used as an affinity probe for hENT1 in human cancer cell lines. Flow cytometry has demonstrated that 5-SAENTA-x8f interacts with NBMPR-binding nucleoside transporters with high affinity ($K_d = 6$ nM).[34,35] In our assay, we equilibrate cells with the affinity probe; equilibrium is complete after 50 min. Cells are then washed to remove nonspecifically bound probe. The probe is released from the cells, separated from cellular debris, and analyzed by capillary electrophoresis, Figure 2, trace b. The component comigrates with 5-SAENTA-x8f, trace c.

Capillary electrophoresis provides a subtle advantage for the analysis of fluorescent probes. Flow cytometry is unable to distinguish between fluorescence of the fluorescein-labeled probe

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and autofluorescence from the cellular matrix. Capillary electrophoresis resolves the autofluorescent component with a migration time of 3.56 min. This peak, which is observed in the 5-SAENTA-x8f-free blank, Figure 2, trace a, is due to endogenous compounds. This autofluorescent component leads to an unstable background signal in flow cytometry, which is particularly problematic when cells with low hENT1 expression levels are analyzed. However, the autofluorescent component is well resolved from the 5-SAENTA-x8f peak in capillary electrophoresis, which improves the precision and accuracy of the assay, particularly for cells with low hENT1 expression level. It is these cells that have the greatest clinical importance, because they are likely to be resistant to chemotherapy treatment with the common nucleoside analogues.

To determine the number of binding sites per cancer cell, the 5-SAENTA-x8f concentration was varied and the amount of bound probe was determined by capillary electrophoresis. A plot of the electrophoresis signal versus the initial 5-SAENTA-x8f concentration in the incubation mix generated a typical binding curve, Figure 3. The 5-SAENTA-x8f signal as a function of initial concentration was saturable, suggesting interaction with a set of high-affinity binding sites. The abundance of NBMPR-binding sites, and therefore hENT1 protein, differs among cell types, and the different cell types used in the experiments of Figure 3 exhibit different binding profiles.[10] The profiles and binding constants obtained in replicate experiments with the same cell line were very similar despite differences in cell numbers (compare Figure 3a and b and Figure 3c and d). Nine aliquots of 435S cells were assayed under conditions that saturated the available binding sites; the relative precision in the number of binding sites was 10%.

Table 1 lists the quantitation results from five cultured human cell lines, along with data obtained by flow cytometry.[12] The Pearson correlation coefficient between hENT1 determination and flow cytometry for these five cell lines is exceedingly strong, $r = 0.96$. The overall assay reproducibility was determined to be 10% from nine independent experiments. The capillary electrophoresis precision was 3 times superior to that of flow cytometry; this improved precision results from elimination of autofluorescence from the background signal in the electrophoresis assay. The instrumental detection limit for 5-SAENTA-x8f is much lower than the number of hENT1 sites in a single cancer cell, which suggests that the analysis of single cells may be possible.

Table 2 lists the K_d values determined from the binding curves. With the exception of the MCF-7 cell line, K_d was ~1 nM; the MCF-7 cell line generated binding constants that were a factor of 3 lower. Capillary electrophoresis is a useful method to obtain binding affinities. These K_d values demonstrate the high-affinity binding between the ligand and the protein, which is the most basic assumption of this quantitation method.

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