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Towards to $hENT_1$-nucleoside transporter selective imaging agents. Synthesis and in vitro evaluation of the radiolabeled SAENTA analogues

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ABSTRACT

Three new potential $hENT_1$ inhibitors suitable for labeling with PET/SPECT radioisotopes were prepared from an advanced intermediate **4**. They were tested for their capability to inhibit binding of SAENTA-fluorescein to HL60 leukemia cells in flow cytometry assay and SAENTA-I (**5**) was determined to be the most active compound. ^{131}I-**5** showed high $hENT_1$-specific binding (up to 54% ID) to 6 from 7 tested tumor cell lines and was chosen for further in vivo study.

Nucleoside analogues are widely used for treatment of different types of tumors. Nucleosides are hydrophilic and can enter cells only by active transport via nucleoside transporters.[1] $hENT_1$-nucleoside transporters[2] belong to the subfamily of concentrative nucleoside transporters, which are characterized by high sensitivity to inhibition with *p*-nitrobenzylmercaptopurine riboside (NBMPR). They play an important role in the intracellular transport of the different nucleosides such as Ara-C, gemcitabine, cytarabine, fludarabine, and others.[3] Several studies showed correlation between sensitivity of different cancers, such as pancreatic, ampullary, lung, and breast cancer towards treatment with nucleoside-based drugs and $hENT_1$ expression level.[4] Moreover, in several cases immunohistochemistry for $hENT_1$ was predictive for patient response on nucleoside chemotherapy.[5] Furthermore, significantly more $hENT_1$ binding sites were found in breast, liver, stomach, and colorectal tumor tissues as in adjacent normal tissues.[6] SAENTA-fluorescein (5′-[(fluorescein-5-ylcarbonyl)-6-aminohexanoyl-(2-aminoethyl)]-N^6-(4-nitrobenzyl)-5′-thioadenosine) and similar fluorescent probes are routinely used to assess the abundance of $hENT_1$ on cell surface by flow cytometry.[7] However, radiotracers suitable for in vivo molecular imaging of $hENT_1$ were not described up to now. Here we describe our preliminary results in this regard.

Originally, a conjugate between 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and SAENTA (DOTA–SAENTA, **1**) was chosen as a promising candidate. Indeed, DOTA conjugates can be labeled with different radiometal ions such as ^{68}Ga, ^{64}Cu, and ^{111}In to enable PET or SPECT imaging. SAENTA derivatives usually have nanomolar affinity to $hENT_1$. They are not internalized and further metabolized by cells and, therefore, can be used to quantify $hENT_1$ transporters localized at the outer leaflet of the cellular membrane which are suitable for the intracellular transport of nucleosides.[8]

Cystamine was doubly acylated with *N*-Boc-protected 6-aminohexanoic acid (BocAhxOH) to give known **2**[9] in 68% yield (Scheme 1). The latter was reduced with tributylphosphane to give the corresponding thiol, which was alkylated directly without purification with 2′-chloroadenosine[10] to furnish **3** in 39% yield on two steps, which in turn was benzylated at 1-position with *p*-nitrobenzylbromide to give after the dimethylamine mediated 1→6 benzyl shift[11] and the acidolytic cleavage of the *N*-Boc-protecting groups an advanced intermediate, H_2N–SAENTA (**4**), as a trifluoroacetate (57% on three steps). Finally, **4** was acylated with DOTA-ONp[12] to give after HPLC purification conjugate **1** in 39% yield. Similarly, acylation of **4** with 5-carboxyfluorescein using EDC/HOBt gave SAENTA-fluorescein[6] (40%), which was used for in vitro study.

DOTA–SAENTA was labeled with ^{111}In (Scheme 1) to give ^{111}In-**1** in 95% radiochemical yield (rcy) and in high radiochemical purity (rp), which was tested for binding to $hENT_1$-positive cells. Surprisingly, we failed to observe significant cell-bound radioactivity in this study. Experiments on competitive binding of **1** and SAENTA-fluorescein to HL60 cells showed, that DOTA–SAENTA as well as In-**1** lacked affinity to $hENT_1$ transporters (data not shown). We reasoned that the absence of binding may be due to the bulkiness and/or the net charge of the macrocyclic chelator.

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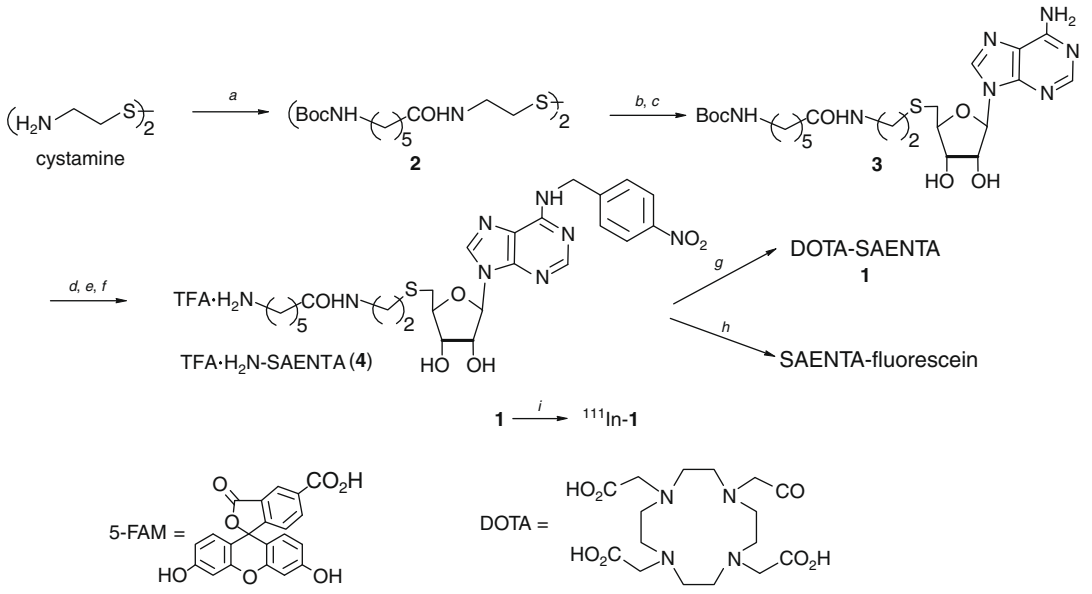
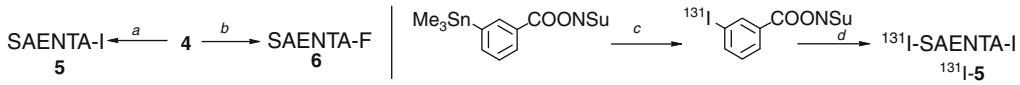


Scheme 1. Reagents and conditions: (a) BocAhxOH, EDC, HOBt, DIEA, CH_2Cl_2, 0 °C to rt, 16 h, 68%; (b) Bu_3P, 90% MeOH, 1 h; (c) MeONa, then 5′-chloroadenosine, reflux, 4 h, 39% on two steps; (d) *p*-NO_2BnBr, mol. sieves 4 Å, DMF, 4 days; (e) Me_2NH in EtOH, 6 h (f) TFA (2.5% TIS, 2.5% H_2O), 0 °C, 10 min, 57% on three steps; (g) DOTAONp, 2,4,6-collidine, MeCN/H_2O, 3 days, 39%; (h) 5-FAM, EDC, HOBt, 2,4,6-collidine, DIEA, DMA, 0 °C to rt, 16 h, 40%; (i) $^{111}InCl_3$, acetate buffer (pH 5.0), 85 °C, 3 min, rcy 95%, rp 95%.



Scheme 2. Reagents and conditions: (a) *m*-IBzOSu, DIEA, 2,4,6-collidine, DMA, 2 days, 28%; (b) *p*-fluorobenzoyl chloride, HOBt, Et_3N, 2,4,6-collidine, CH_2Cl_2/DMF, 16 h, 0 °C to rt, 44%; (c) $Na^{131}I$, chloroamine T, MeOH/AcOH, 5 min; (d) **4**, borate buffer (pH 8), 2 h, rcy 22% two steps, rp > 98%.

Accordingly, we prepared SAENTA derivatives with small neutral and lipophilic side chain. Compound **4** was acylated with *N*-succimidyl *m*-iodobenzoate (*m*-IBzOSu) or prepared in situ 1-benzotriazolyl *p*-fluorobenzoate to give SAENTA-I (**5**) and SAENTA-F (**6**) in 28% and 44% yield, respectively (Scheme 2). They were tested regarding inhibition of the $hENT_1$-specific SAENTA-fluorescein binding to HL60 leukemia cells by flow cytometry. Gratifyingly, **5** and **6** inhibited SAENTA-fluorescein binding to HL60 cells (Fig. 1) with **5** as a more potent compound. It led to full inhibition of SAENTA-fluorescein binding at equimolar concentration. The IC_{50} values (concentration of inhibitor that inhibited 50% of SAENTA-fluorescein binding) 1.4 ± 1.0 nM and 5.1 ± 1.0 nM for 5 and 6,

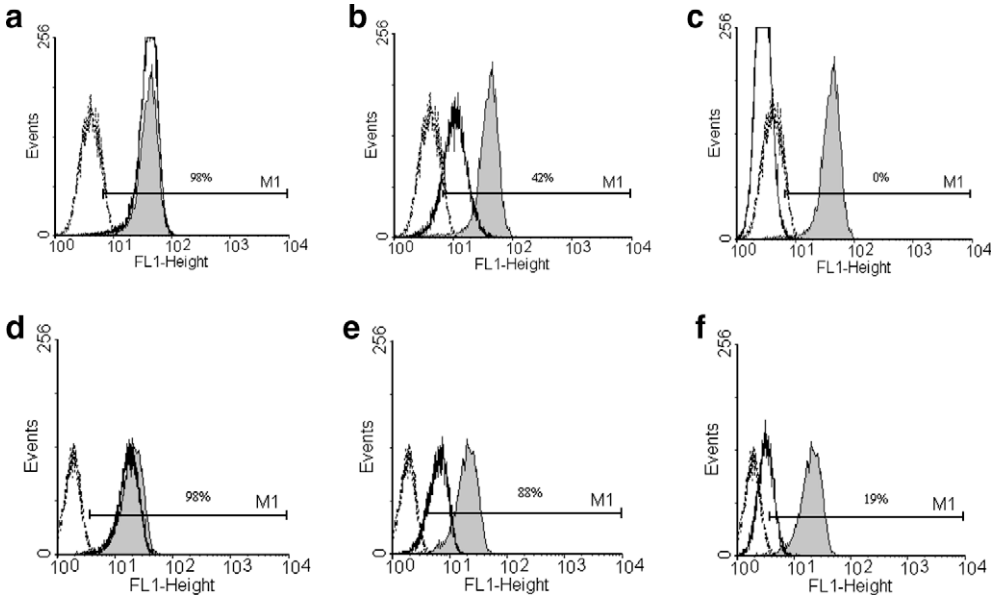


Figure 1. Flow cytometric competition studies of binding of SAENTA-fluorescein with SAENTA-I (**5**) (a–c), and with SAENTA-F (**6**) (d–f) using HL60 cells. The binding of SAENTA-fluorescein (50 nM) was examined in the presence of **5**: 5 nM (a), 25 nM (b), 50 nM (c) or in the presence of **6**: 5 nM (d), 25 nM (e), 50 nM (f). Dashed lines—negative control; bold lines—binding of SAENTA-fluorescein preincubated with **5** or **6**; filled histogram—binding of SAENTA-fluorescein.

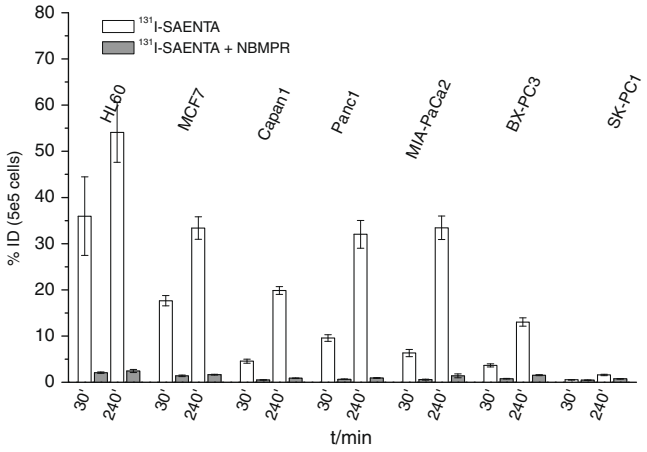


Figure 2. Cell binding of ^{131}I-**5** (20 KBq/5 × 10^5 cells) to HL60 leukemia, MCF7 breast, and Capan1, Panc1, MIA-PaCa2, BX-PC3, SK-PC1 pancreas tumor cells depending on pre-incubation with NBMPR (7.2 μM).

Table 1
K_D and B_{max}[a] for binding of SAENTA-fluorescein to the used cell lines

Cell line	K_D[b] (nM)	B_{max}[b,c] (MFU)
HL60	0.26 ± 0.05	21.1 ± 0.7
MCF7	n.d.[d]	n.d.[d]
Capan1	0.32 ± 0.08	9.5 ± 0.4
Panc1	3.8 ± 0.4	49.8 ± 1.7
MIA-PaCa2	0.91 ± 0.07	30.7 ± 0.6
BX-PC3	0.36 ± 0.05	6.7 ± 0.2
SK-PC1	0.40 ± 0.28	2.6 ± 0.4

[a] K_D—apparent equilibrium dissociation constant for SAENTA-fluorescein at es nucleoside transporter sites; B_{max}—maximum specifically bound SAENTA-fluorescein per 10^4 cells.
[b] K_D and B_{max} values were determined according to Ref. 16.
[c] MFU—mean fluorescence units.
[d] n.d.—not determined.

respectively, were obtained by the nonlinear regression analysis of the inhibitor concentration–fluorescence curves using GraphPad Prism software. Inhibition constants (K_i) were calculated from IC_{50} using the Chaeng–Pristoff equation:[13] $K_i = IC_{50}/(1 + [S]/K_D)$, where [S]—concentration of SAENTA-fluorescein, K_D—equilibrium dissociation constant for SAENTA-fluorescein at *es* nucleoside transporter sites. The K_i values 0.018 ± 0.016 nM and 0.064 ± 0.025 nM were obtained for **5** and **6**, respectively, demonstrating somewhat stronger binding for **5**. The K_i values were even lower than that of NBMPR [K_i(NBMPR) = 0.1–1.0 nM].[13a,14] The difference between the K_i values of **5** and **6** indicates that in case of SAENTA derived compounds even minor structural changes in the substituent at the 5′ position of adenosine residue can affect the binding to ENT_1 transporters.

^{131}I-**5** was prepared by acylation of **4** with ^{131}I-*m*-IBzOSu, which in turn was obtained by radioiodination of the stannyl precursor[15] (Scheme 2) in 22% radiochemical yield on two steps (Scheme 2). It was tested for binding to leukemia, breast, and pancreas tumor cell lines and showed good or moderate time-dependent binding to 6 from 7 tested cell lines (Fig. 2). The cell binding was inhibited up to >90% by NBMPR demonstrating its $hENT_1$ specificity. Additionally, cell binding of ^{131}I-**5** correlated significantly ($P < 0.05$) with the binding potential (B_{max}/K_D) of the ENT_1-specific SAENTA-fluorescein binding (Fig. 3). The respective binding parameters, the binding affinity (K_D), and the capacity (B_{max}), were determined

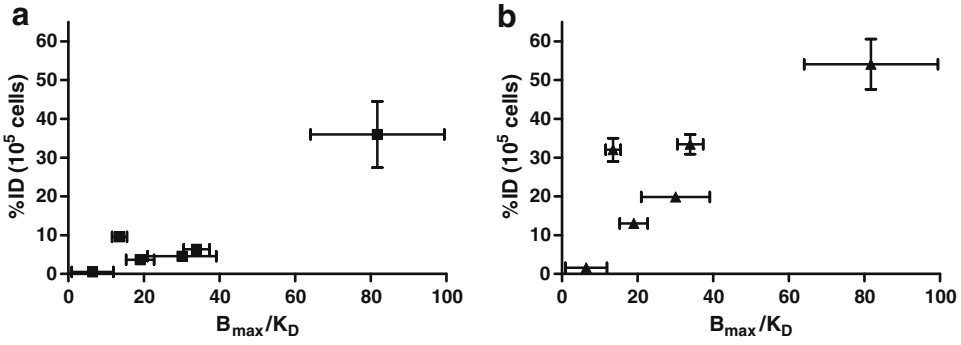


Figure 3. Correlation between the binding potency of SAENTA-fluorescein and the cell binding of ^{131}I-SAENTA-I (^{131}I-**5**). The following parameters were obtained: (a) after 30 min incubation: slope of linear regression = 0.45 ± 0.09, $P = 0.008$, $R^2 = 0.86$; (b) after 240 min incubation: slope of linear regression = 0.58 ± 0.18, $P = 0.03$, $R^2 = 0.72$. Error bars smaller than symbols are not shown.

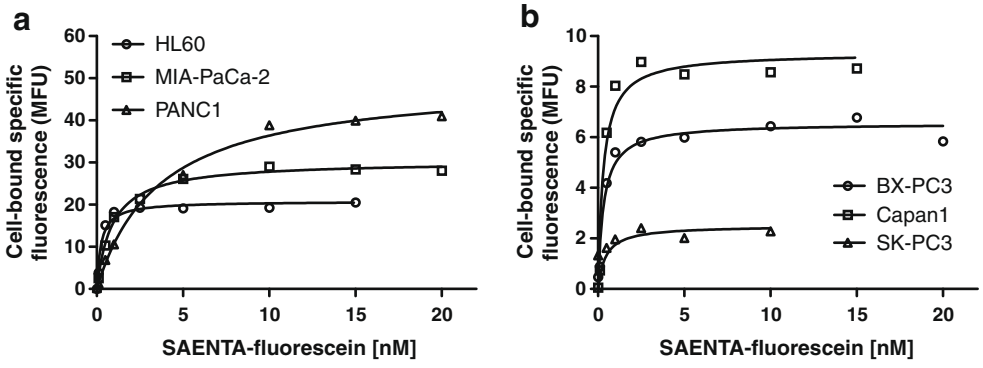


Figure 4. Flow cytometric analysis of the dependence on concentration of ENT_1-selective binding of SAENTA-fluorescein. The specific binding data represent the difference between the total and nonspecific fluorescence. The nonspecific fluorescence was measured in presence of 7.2 μM NBMPR.

by flow cytometry (Table 1, Fig. 4) using the Prism Software. At present we cannot explain variations of the K_D values which suggest differences in affinity of the SAENTA-fluorescein binding sites by different cell lines.

In conclusion, SAENTA-I showed consistently high and $hENT_1$-specific binding to most tumor cell lines tested. SAENTA-I is therefore a $hENT_1$-selective marker which should be further studied for its suitability for imaging the expression of $hENT_1$ nucleoside transporters in vivo and, if successful, on its capability to predict tumor sensitivity towards nucleoside-based chemotherapeutic agents.

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