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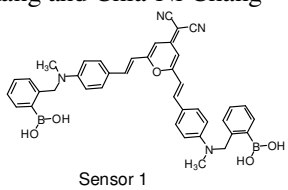

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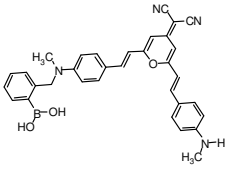

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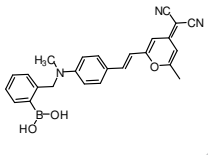

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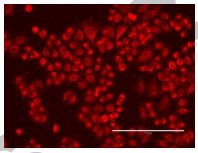

Sensor 1-HepG2

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Synthesis of Three Fluorescent Boronic Acid Sensors for Tumor Marker Sialyl Lewis X in Cancer Diagnosis

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ABSTRACT

Sialyl Lewis X (sLex) is a carbohydrate that is considered not only a marker for cancer, but also an antigen associated with the malignant behavior of cancerous cells. We have synthesized three fluorescent boronic acid sensors as potential sensors for sLex. Photoinduced electron transfer by fluorescence analyzer was used to assess sensor-sLex antigen binding. The reaction was carried out in mixed aqueous solution, and Sensor 3 was identified as showing the strongest fluorescence enhancement upon binding to sLex at 10 nM. In cell-line culture experiments, Sensor 1 was shown to label sLex expression positively for HepG2 , Colo 205, and COS-7 cells, and negatively for MDA-MB-231 cells; Sensor 2 did so positively for HepG2, PLC/PRF/5, and Colo 205 cells, and negatively for MDA-MB-231 and COS7 cells; and Sensor 3 did so positively for PLC/PRF/5 and HepG2 cells, and negatively for MDA-MD-231 and COS7 cells. MTT cytotoxicity experiments results showed that the three sensors are nontoxic, and Hoechst 33258 experiments showed that no apoptosis occurred

Introduction

Glycan molecules such as sialyl Lewis X (sLex; Fig 1) and sialyl Lewis A (sLea) have often been associated with the development of cancerous diseases. sLex is a tetrasaccharide located at the terminus of a glycan chain and, because of this terminal positioning, sLex epitopes are often used as clinical indicators for serum tumor markers.[1-3] Expression of these glycan molecules in cancer cells is significantly higher than in non-malignant epithelial cells. Over-expression of the sLex antigen has been found to be associated with colorectal, bladder, stomach, and gastrointestinal cancers. Increased expression of sLex has been shown to be correlated with strong adherence of cancer cells to E-selectin on vascular endothelial cells.[4-7] Moreover, sLex is also a key component of the carbohydrate ligands for P- and L-selectins.[8] Together, these results indicate that sLex plays an important role in hematogenous cancer metastasis.

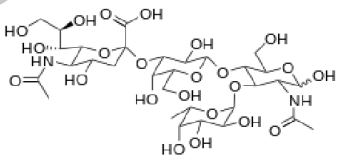
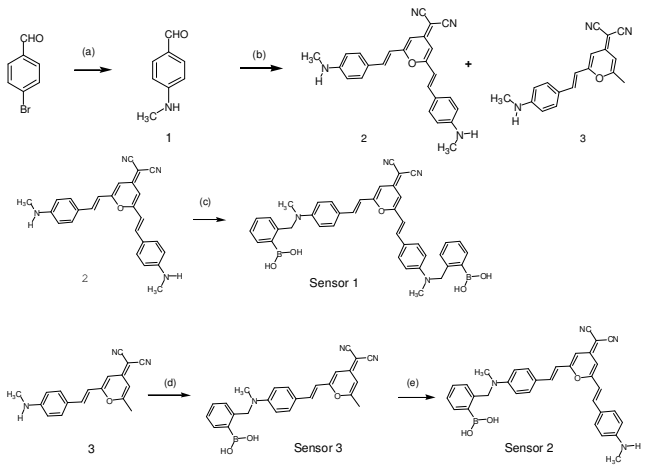


Fig 1. Sialyl Lewis X tetrasaccharide

A typical photoinduced electron transfer (PET) sensor[9] is composed of three main components: a fluorophore, a spacer, and a receptor. The fluorophore consists of a conjugated system that will fluoresce when excited by UV visible light. The spacer consists of an electron-rich group, such as nitrogen, that will readily transfer an electron to the fluorophore, thereby quenching fluorescence. When the receptor is positioned in the segment of the sensor that can directly bind to the target analyte, sLex, the fluorescence-intensity increase upon binding is thought to be due to the diminished fluorescence quenching by the transfer of unbound nitrogen electrons during boronate ester formation. The magnitude of the increase in fluorescence intensity can be measured using a fluorescence analyzer. With this design in mind, we synthesized three fluorescent boronic acid sensors with appropriate bond length and structure to match the epitope of sub-terminal tetrasaccharides on sLex. When the probe binds strongly to the target sLex antigen through the boric acid moieties, it will exhibit high fluorescence intensity enhancement. The development of sensors to recognize sLex is invaluable for the diagnosis and early detection of cancers.

Results and Discussion

Synthesis of the three fluorescent boronic acid sensors (Scheme 1) commenced by Ullmann amination[10] of commercially available 4-bromobenzaldehyde with 5 mole % copper powder in aqueous methylamine at 100ºC. This produced an 89% yield of 4-(methylamino)benzaldehyde (**1**). The simplicity of the Knoevenagel condensation of aldehyde (**1**) with (2,6-dimethyl-4H-pyran-4-ylidene)malononitrile[11-12] in the presence of base led to the production of dialkylation (**2**) and monoalkylation compounds (**3**) in yields of 73% and 26%, respectively. The free amines (**2**) and (**3**) were then reacted separately with 2-(bromomethyl)phenylboronic acid in the presence of potassium carbonate in acetonitrile to produce a 94% yield of the diboronic acid **Sensor 1** and 82% yield of the monoboronic acid sensor **3**. **Sensor 3** was condensed with 4-(methylamino)benzaldehyde in the presence of piperidine in acetonitrile to produce an 89% yield of **Sensor 2**.



Scheme 1. (a) (i) MeNH2, Cu (89%); (b) (2,6-Dimethyl-4H-pyran-4-ylidene)malononitrile, CH3CN 2 (73%) 3 (26%); (c) 2-(bromomethyl)phenylboronic acid, K2CO3 (94%); (d) 2-(bromomethyl)phenylboronic acid, K2CO3 (82%); (e) 4-(methylamino)benzaldehyde (89%).

The three sensors synthesized – Sensors 1, 2, and 3 – had emission spectra with impressive red fluorescence with a peak climax at 617, 614, and 607 nm (400 nm excitation) in methanol, respectively. Fluorescence binding experiments of the boronic acid sensors with sLex were thus prepared and conducted in a mixture of MeOH and PBS (0.1 M phosphate buffer solution, pH 7.4) (1:1, v/v)[13] The concentration of sensors was fixed at 1 μM, 0.1 μM, and 0.01 μM, while the concentration of sLex was set at 60 μM. Based on the principles of PET, binding of the boronic acid sensors with the target sLex should increase their respective fluorescence intensities. As expected, we observed increases in the fluorescence intensities of each sensor. The profile of fluorescence intensity change was examined using a fluorescence analyzer**.** A favorable interaction or close integration of the probe sensor with the sLex antigen was observed when the fluorescence intensity of all three sensors is greater than 200% at a sensor concentration of 1 μM. Sensor 3 was found to exhibit the strongest fluorescence enhancement upon binding with sLex at 0.10 μM (300%) and 0.01 μM (120%) (Fig 2).

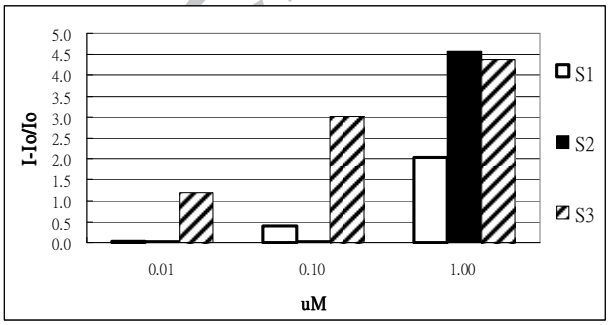


Fig 2. Fluorescence binding studies by the fluorescence analyzer test.

Due to the high fluorescent enhancement of Sensor 1, Sensor 2, and Sensor 3 upon binding with sLex, it was necessary to assess the effect of the sensors in cell culture using sLex-expressing cells such as hepatocellular carcinoma cell-lines (HepG2 and PLC/PRF/5). The positive expression of sialyl Lewis X in SMMU-7721, PLF/PRF/5 and HepG2 cell lines was 7.03%, 63.35%, and 97.29%, respectively. Breast cancer cell line MDA-MB-231 (for which CD44 is positively expressed in 90.19% of cells according to FACS analysis). Colon cancer cell line Colo 205 carrying sialyl Lewis a and x epitopes (H-CanAg) were purified by trichloroacetic acid precipitation and Superose 6 gel filtration. Colo-205 expressed high levels of sLex/sLe. COS7 was used as control, because it does not express sLex, Lea, Leb, or Ley. Cells were incubated with 10 μM of Sensor 1, Sensor 2, or Sensor 3 in six-well plates in the dark at 4ºC. After 45 min, cells were observed using a fluorescence microscope in accordance with Weston and Wang.[14] Sensor 1 showed close integration with hepatocellular carcinoma cell-line HepG2 and with colon cancer cell-line Colo 205, and with Cos-7; Sensor 2 showed close integration with hepatoma B cell-line PLC/PRF/5, with HepG2, and with Colo 205; Sensor 3 showed specific integration with PLC/PRF/5 and with HepG2 (Fig 3). MTT cytotoxicity experiments results showed all three sensors are nontoxic, with the cell viability of HepG2 or PLC/PRF/5 cells after action of the three sensors remaining at nearly 100% (Fig 4). Hoechst 33258 experiments showed no occurrence of apoptosis (Fig 5).

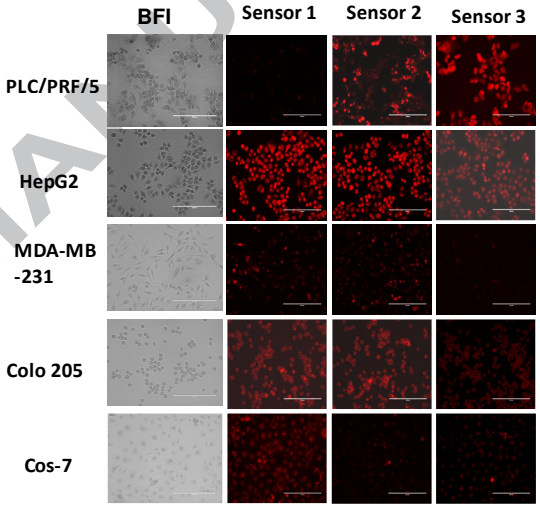


Fig 3. Fluorescent labeling studies of PLC/PRF/5, HEPG2, MDA-MB-231, Colo 205, and COS-7 cells by fluorescent microscopy.

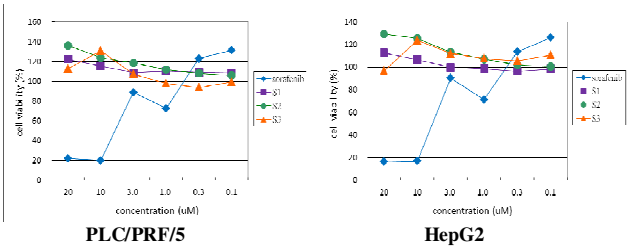


Fig. 4 Cell viability of three sensors acting on HepG2 or PLC/PRF/5 cells

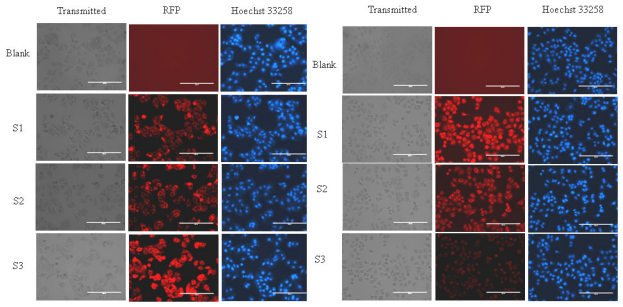


Fig 5 Fluorescent labeling studies and Hoechst 33258 experiments on PLC/PRF/5 and HepG2 cells

Conclusion

In conclusion, we synthesized three fluorescent boronic acid sensors capable of targeting sLex-expressing cells. Fluorescence spectroscopy showed high intensity enhancement over a wide range of concentrations. The results of fluorescence spectroscopy indicated that three different types of cell lines expressing sLex antigen showed close integration with the synthesized sensors. Sensor 2 is the better choice for identifying both positive and negative expression, and thus it has great potential for use in cancer diagnosis

Acknowledgement

Financial support from the Innovation Panel of the Industrial Technology Research Institutes is gratefully acknowledged.

Experimental

4-(Methylamino)benzaldehyde (1)
A mixture of 4-bromobenzaldehyde (1.85 g, 10 mmol), 40% aqueous methylamine solution (5.4 mL, 50 mmol), copper powder (32 mg, 0.5 mmol), and a stirring bar was sealed in a 100-mL screw-top tube and stirred in an oil bath at 100ºC. After a 17-hour incubation period, the reaction mixture was cooled to room temperature and ethyl acetate (20 mL) was added to extract the aryl amine. The organic layer was separated and the aqueous layer was extracted using ethyl acetate (3 x 10 mL). The combined extracts were then dried by anhydrous sodium sulfate and the solvent was removed under reduced pressure to give a crude product that was purified by silica gel column chromatography EtOAc/n-hexane (1:1) to give the pure product 4-(methylamino)benzaldehyde (1.21 g, 89%, yellow crystals). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 9.70 (s, 1H), 7.68 (d, 2H, *J*=8.5 Hz), 6.59 (d, 2H, *J*=8.5 MHz), 4.45 (s, 1H), 2.90 (s, 3H), ^{13}C-NMR (125 MHz, $CDCl_3$) δ 190.29, 154.32, 132.21, 126.02, 111.34, 29.85, ESI-MS (positive ion) m/z: 136.2 $[M+H]^+$, 158.4 $[M+Na]^+$.

(2) and (3)
A mixture of 2,6-Dimethyl-4H-pyran-4-ylidene)malononitrile (1.98 g, 11.4 mmol), 4-(methylamino)benzaldehyde **(1)** (1.25 g, 9.12 mmol), and piperidine (1 mL) in acetonitrile (50 mL) was heated to reflux under argon overnight. The reaction was then cooled and the insoluble material was filtered off to give dialkylation compound **2** (1.92 g, 73%). The filtrate was then evaporated to dryness under reduced pressure. Dichloromethane was added to dilute the residue and filtered to give monoalylation compound **3** (0.99 g, 26%). **Dialkylation (2)** ^{1}H-NMR (500 MHz, d6-DMSO) δ 7.61 (d, 2H, *J*=16Hz), 7.57 (d, 4H, *J*=8Hz), 6.98 (d, 2H, *J*=16Hz), 6.64 (s, 2H), 6.58 (d, 4H, *J*=8.5Hz), 6.47 (dd, 2H, *J*=4.5, 5Hz), 2.74 (d, 6H, *J*=5Hz), ^{13}C-NMR (125 MHz, d6-DMSO) δ 159.68, 155.56, 151.65, 138.49, 129.95, 122.04, 116.16, 112.09, 111.31, 104.30, 52.64, 29.00, ESI-MS (positive ion) m/z: 407.2 $[M+H]^+$, HRMS (ESI) 407.1872 (407.1866 calculated for $C_{26}H_{23}N_4O$). **Monoalkylation (3)** ^{1}H-NMR (500 MHz, d6-DMSO) δ 7.45 (d, 2H, *J*=9Hz), 7.40 (d, 1H, *J*=16Hz), 6.95 (d, 1H, *J*=16Hz), 6.70 (d, 1H, *J*=2.5H), 6.60 (d, 1H, *J*=1Hz), 6.55 (d, 2H, *J*=8.5Hz), 6.47 (q, 1H, *J*=5Hz), 2.72 (d, 3H, *J*=5Hz), 2.41 (s, 3H), ^{13}C-NMR (125 MHz, d6-DMSO) δ 163.81, 161.43, 156.75, 152.21, 139.16, 130.27, 122.22, 116.05, 112.23, 111.83, 105.59, 104.93, 54.07, 29.43, 19.54, ESI-MS (positive ion) m/z: 290.2 $[M+H]^+$, HRMS (ESI) 290.1284 (290.1288 calculated for $C_{18}H_{16}N_3O$).

Sensor 1
A mixture of dialkylation compound **(2)** (29.4 mg, 73 µmol), 2-(bromomethyl)phenylboronic acid (128.91 mg, 0.60 mmol), and potassium carbonate (200 mg, 1.46 mmol) in dry acetone (10 mL) was stirred at room temperature for 48 hours. The insoluble materials were filtered off and the filtrate was evaporated in *vacuo* and purification was carried out by HPLC using a preparative column, YMC-Pack ODS-A 250 x 20 mm, and mobile phase of acetonitrile/water (8:2), rate=8 mL/min, retention time 16.10 min, yielded **Sensor 1** in 94% (45.8 mg, 68.0 mmol). ^{1}H-NMR (500 MHz, d6-acetone) δ (ppm): 7.68 (d, 4H, *J*=8Hz), 7.56 (d, 4H, *J*=8.5Hz), 7.32 (s, 4H), 7.26 (t, 2H, *J*=8Hz), 7.18 (t, 2H, *J*=7Hz), 7.06 (d, 2H, *J*=7.5Hz), 6.93 (d, 2H, *J*=16Hz), 6.79 (d, 4H, *J*=9Hz), 6.56 (d, 2H, *J*=1.5Hz), 4.86 (s, 4H), 3.09 (s, 6H), ^{13}C-NMR (125 MHz, d6-Acetine) δ 161.03, 157.16, 152.71, 143.94, 139.59, 135.70, 130.91, 130.64, 127.01, 126.72, 124.53 ,116.81, 114.50, 113.70, 106.00, 57.44, 56.38, 39.49, ESI-MS (positive ion) m/z: 675.2 $[M+H]^+$, 731.2 $[M+4MeOH-4H_2O+H]^+$, HRMS (ESI) 675.2964 (675.2958 calculated for $C_{40}H_{37}B_2N_4O_5$).

Sensor 3
The same purification conditions were used with HPLC using a preparative column, YMC-Pack ODS-A 250 x 20 mm, and mobile phase, acetonitrile/water (6:4), rate=8 mL/min, retention time 11.66 min, yielded **Sensor 3** in 84%. ^{1}H NMR (500 MHz, d6-DMSO) δ (ppm): 8.16 (s, 2H), 7.54 (d, 1H, *J*=6.5Hz), 7.50 (d, 2H, *J*=9Hz), 7.42 (d, 1H, *J*=16Hz), 7.25 (t, 1H, *J*=6.5Hz), 7.19 (t, 1H, *J*=6.5 z), 7.00 (d, 1H, *J*=16Hz), 6.94 (d, 1H, *J*=7.5Hz), 6.73 (s, 2H), 6.71 (s, 1H), 6.61 (s, 1H), 4.78 (s, 2H), 3.07 (s, 3H), 2.42 (s, 3H), ^{13}C-NMR (125 MHz, d6-DMSO) δ (ppm): 163.91, 161.28, 156.78, 151.23, 141.92, 138.70, 134.01, 130.04, 129.16, 125.90, 124.99, 122.54, 116.02, 113.06, 112.19, 105.65, 105.21, 55.75, 54.26, 19.55, ESI-MS (positive ion) m/z: 424.2 $[M+H]^+$, 438.3 $[M+MeOH-H2O+H]^+$, 452.3 $[M+2MeOH-2H2O+H]^+$, HRMS (ESI) 424.1827 (424.1831 calculated for $C_{25}H_{23}BN_3O_3$).

Sensor 2
A mixture of sensor 3 (20.4 mg, 48 µmol), 4-(methylamino)benzaldehyde (7.4 mg, 54 µmol), and piperidine (0.1 mL) in acetonitrile (12 mL) was heated to reflux under argon overnight. The reaction was then cooled and the mixture evaporated to dryness under reduced pressure. Purification was carried out using HPLC using a preparative column, YMC-Pack ODS-A 250 x 20 mm, and mobile phase of acetonitrile/water (8:2), rate=8 mL/min, retention time 18.26 min), yielded **Sensor 2** in 89% (23.1 mg, 42 µmol). ^{1}H-NMR (500 MHz, d6-Acetone) δ (ppm): 7.78-7.73 (m, 2H), 7.65 (q, 4H, *J*=8.5 Hz), 7.43 (s, 2H), 7.35 (t, 1H, *J*=8Hz), 7.27 (t, 1H, *J*=7Hz), 7.15 (d, 1H, *J*=8Hz), 7.03 (d, 1H, *J*=18.5Hz), 7.00 (d, 1H, *J*=18.5Hz), 6.85 (d, 2H, *J*=8.5 Hz), 6.72 (d, 2H, *J*=8.5Hz), 6.66 (s, 3H), 4.90 (s, 2H), 3.13 (s, 3H), 2.08 (s, 3H), ^{13}C-NMR (125 MHz, d6-acetone) δ (ppm): 161.21, 161.05, 157.22, 153.30, 152.72, 143.98, 140.00, 139.60,

135.74, 131.18, 130.92, 130.65, 127.01, 126.71, 124.55, 124.28, 116.84, 114.53, 113.86, 113.71, 112.98, 105.99, 105.80, 57.45, 55.19, 39.51. ESI-MS (positive ion) m/z: 541.1 $[M+H]^+$, HRMS (ESI) 541.2406 (541.2411 calculated for $C_{33}H_{30}BN_4O_3$).

Determination of cytotoxicity

HepG2 or PLC/PRF/5 cells were seeded in 96-well plates at $1x10^4$ cells/well, and incubated at 37°C in a 5% CO_2 incubator. After 24 h, the culture supernatant was replaced with fresh DMEM and different concentrations of Sensor 1, 2, or 3. The plates were incubated for 48 h. Then, 10 µL of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at a concentration of 5 mg/mL was added to each well and incubated for 4 h at 37°C in the 5% CO_2 incubator. The culture medium was removed and 100 µL DMSO were added and incubated at room temperature for 30 min. The absorbance of each well was measured by FlexStation 3 microplate reader at a wavelength of 595 nm. Cell viability was calculated as nearly 100%, employing 1:1 methanol/PBS-treated cells as the 100% viable control and using the following formula: (A595 of sensor-treated samples/A595 of control)x100.

Fluorescent Labeling Studies

Six-well plates were seeded with $5x10^5$ cells per well and incubated at 37°C and 5% CO_2 for 48 h. The media was removed and cells were washed twice with PBS. The cells were fixed with 1.5 ml of 1:1 methanol/PBS and incubated 20 min at 4°C. After incubation, the methanol/PBS solution was removed and cells were washed twice with PBS. Sensors 1, 2, and 3 were diluted in 1:1 methanol/PBS and added to wells at concentrations of 1.0–10 µM. One well was incubated only in methanol/PBS without a compound, as a negative control. The plates were then incubated in darkness at 4°C for 45 min. For nuclear staining, cells were subsequently washed with PBS and chromatin was visualized by exposure to Hoechst 33258 (10 µg/ml). Plates were examined with phase contrast microscopy followed by fluorescent microscopy (RFP wavelengths: 531 nm excitation, 593 nm emission); 20X lens plates were photographed using a high-sensitivity monochrome, 1360x1024, 6.45 µm/pixel camera (Sony® ICX285AL CCD).

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Supplementary Material

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