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A novel near-infrared fluorescence imaging probe that preferentially binds to cannabinoid receptors CB₂R over CB₁R

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Abstract

The type 2 cannabinoid receptors (CB₂R) have gained much attention recently due to their important regulatory role in a host of pathophysiological processes. However, the exact biological function of CB₂R and how this function might change depending on disease progression remains unclear and could be better studied with highly sensitive and selective imaging tools for identifying the receptors. Here we report the first near infrared fluorescence imaging probe (**NIR760-XLP6**) that binds preferentially to CB₂R over the type 1 cannabinoid receptors (CB₁R). The selectivity of the probe was demonstrated by fluorescence microscopy using DBT-CB₂ and DBT-CB₁ cells. Furthermore, in mouse tumor models, **NIR760-XLP6** showed significantly higher uptake in DBT-CB₂ than that in DBT-CB₁ tumors. These findings indicate that **NIR760-XLP6** is a promising imaging tool for the study of CB₂R regulation.

Keywords

cannabinoid; CB2 receptor; fluorescence imaging; cancer; near infrared

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1. Introduction

The endogenous cannabinoids system includes cannabinoid receptors and their endogenous ligands that have been studied extensively due to its implication in many biological process such as the control of membrane potential,[1] metabolic regulation,[2] cell proliferation[3] and many other cell function in both the central nervous system and peripheral tissues.[4] These effects are mainly mediated via two subtypes of cannabinoid receptors: type 1 cannabinoid receptor (CB₁R) and type 2 cannabinoid receptor (CB₂R).[5] Both receptors are G protein-coupled, membrane-bound proteins that regulate multiple signaling cascades, including adenylyl cyclase and mitogen protein kinases.[6] CB₁R is primarily found in the central nerve system, and CB₂R is mainly present in immune cells, although recent studies indicate that CB₁R also express in a number of peripheral tissues and CB₂R may also exists in the central nervous system.[7] A large amount of efforts have been invested to study CB₂R function and this target is now considered to hold major therapeutic promises. For example, studies have shown that agonists acting on CB₂R may exhibit potential role in cancer treatment.[8-10] Other studies have highlighted the roles for CB₂R in inflammatory and neuropathic pain models.[11] Some neuroprotective roles of inflammation in nervous system have also been associated with CB₂R that could lead to the prevention of select neurodegenerative diseases.[12] However, the precise expression pattern of CB₂R and the number of receptors that need to be activated to regulate these disease processes remains unclear.[13] Therefore, there is an urgent need to develop reliable imaging tools to answer these important questions about CB₂R.

As a low-cost imaging method with high sensitivity and resolution, fluorescence imaging is widely used to study receptors at the cellular and molecular levels.[14-16] For in vivo imaging studies, near infrared (NIR) light (650-900 nm) is usually adopted, owing to the low tissue absorption and negligible tissue autofluorescence in the NIR region.[14] However, it is difficult to develop CB₂R-targeted fluorescent probes because conjugating a relatively large fluorescent dye to a CB₂R ligand could obliterate its binding to CB₂R.[17] In fact, even attaching a small linker to a CB₂R ligand often leads to the loss of binding.[18] We recently overcame the difficulty and introduced NIR dyes to strategic positions of a pyrazole and quinolone structure respectively.[19-23] The specificity of these probes was demonstrated by blocking studies both in vitro and in vivo. Despite the recent progress made on developing CB₂R-targeted fluorescent probes, one key issue of CB₂R imaging is their selectivity. Because both CB₁R and CB₂R are expressed by the same cells in certain physiological and pathophysiological conditions,[24, 25] and these receptors share high degrees of homology in their binding sites, it is critical to develop probes that preferably bind to CB₂R over CB₁R. Moreover, CB₂R-targeted imaging probes with significantly lower CB₁R binding affinity are unlikely to elicit undesired psychotropic effects linked to CB₁R activation.[14-16] So far, no selective CB₂R fluorescent probe has been reported. This is not surprising because in addition to the difficulty in developing CB₂R-targeted fluorescent probe as discussed above, a CB₂R-selective fluorescent probe has to overcome the challenge of recognizing CB₂R from CB₁R which share significant sequence homology (CB₁R and CB₂R share an overall 44% amino acid homology and a 68% amino acid homology within the transmembrane domains).[26] Here we report the first NIR

fluorescence imaging probe, **NIR760-XLP6**, which shows preferable binding to CB₂R over CB₁R both in vitro and in vivo.

2. Materials and Methods

All animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the Medical Research Council of University of Pittsburgh.

2.1 Materials

All solvents and reagents were used as obtained from Sigma Aldrich (St. Louis, MO) and Thermo Fisher Scientific (Pittsburgh, PA), unless otherwise stated. 3-Bormo-5-aminopyrazole from Ark Pharm, Inc. (Libertyville, IL). 0.1% Triton X-100 in PBS, SR-141716A, indocyanine green (ICG), and DAPI from Sigma-Aldrich (St. Louis, MO). 4% paraformaldehyde in PBS from Affymetrix (Santa Clara, CA). Doxorubicin from A Chemtek Inc. (Worcester, MA). CellTiter-Glo® Luminescent Cell Viability Assay kit from Promega (Madison, WI). RNazol Reagent, and Platinum Taq DNA polymerase from Invitrogen (Carlsbad, CA). Primers for CB₂R, CB₁R and GAPDH (housekeeping gene) from Integrated DNA Technologies (Coralville, IA). ProLong® Gold antifade reagent from Life Technologies (Carlsbad, CA). DRAQ-5 from Cell Signaling Technology (Beverly, MA). Transfected mouse delayed brain tumor (DBT) cell line that expresses CB₂R (DBT-CB₂) or CB₁R (DBT-CB₁). 6 - 8 week old female nu/nu mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

2.2. Measurements and analysis

¹H and ¹³C NMR spectra were recorded at 25 °C on the Bruker Avance III 400 MHz. Unless otherwise specified, chemical shifts δ were expressed in parts per million (ppm) based on the residual solvent signal in DMSO-*d*₆ (δ 2.50 ppm) or tetramethylsilane (TMS) in CDCl₃ (δ 0.00 ppm), and coupling constants *J* are given in Hz. Coupling patterns are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), ABq (AB quartet), and m (multiplet). Electrospray mass spectra were recorded on a Waters LCT Premier mass spectrometer in positive ion mode. Reverse phase HPLC was performed using Waters HPLC system equipped with Waters-1525 solvent delivery systems and Waters 2998 UV detector. Separation was achieved on a semi-preparative Phenomenex (Torrance, CA) Jupiter® 5 μ m C18 300Å column (5 micron, 250 × 10.00 mm, Part Number: 00G-4053-N0) at a flow rate maintained at 4.0 mL/min. A solvent system composed of water containing 0.1% TFA as solvent A and acetonitrile containing 0.1% TFA as solvent B was used over the stated gradient. The column effluent was monitored with a UV/Vis detector set at 760 nm.

2.3. Synthesis of NIR760-XLP6

Compound **1** was prepared according to literature[27] from 3-bormo-5-aminopyrazole. The **NIR760** dye was prepared using the previously described procedure.[21, 28]

2.3.1. Synthesis of 2—Under Argon atmosphere, a mixture of pyrazolopyrimidine derivative **1** (1.4 g, 5.0 mmol) and anhydrous potassium carbonate (2.8 g, 20 mmol) in

anhydrous DMF (40 mL) was heated at 100 °C. After 1 h, 1-bromopentane (1.5 g, 10 mmol) was added and the mixture was stirred at 100 °C for 23 h. After being cooled down to room temperature, the solution was evaporated to dryness *in vacuo*. The residue was triturated with cold water (50 mL) and filtered. The white precipitate formed was washed with cold water (50 mL) and dried *in vacuo*. The crude product was purified by column chromatography using a gradient eluent of ethyl acetate/hexanes = 2 : 1 to 4 : 1 to give the titled compound **2** as a white solid (1.3 g, 74%); ¹H-NMR (400 MHz, CDCl₃, TMS): δ 0.93 (t, 3H, *J* = 7.0 Hz), 1.38 (7H, m), 1.89 (quin, 2H, *J* = 7.5 Hz), 4.00 (t, 2H, *J* = 7.4 Hz), 4.38 (q, 2H, *J* = 7.1 Hz), 6.19 (s, 1H), 8.29 (s, 1H). ¹³C-NMR (100 MHz, CDCl₃, TMS): δ 13.8, 14.3, 22.1, 27.9, 28.5, 54.9, 61.5, 93.9, 101.3, 133.6, 142.2, 146.8, 151.4, 163.7. MS (ESI): *m/z* = 356.062, calcd. for C₁₄H₁₉N₃O₃Br *m/z* (M+H)⁺ = 356.061.

2.3.2. Synthesis of 3—To a solution of pyrazolopyrimidine derivative **2** (1.1 g, 3.0 mmol) in methanol (10 mL) was added 10% sodium hydroxide aqueous solution (10 mL). The resulting solution was stirred at 25 °C for 1 h followed by addition of 10% hydrochloric acid aqueous solution to adjust the pH to 4. The precipitate formed was collected by filtration, washed with water (10 mL) and dried *in vacuo* to give the titled compound **3** as a white solid (0.91 g, 92%). The product was used in the next step without further purification; ¹H-NMR (400 MHz, DMSO-*d*₆): δ 0.85 (t, 3H, *J* = 6.9 Hz), 1.22-1.34 (m, 4H), 1.74 (quin, 2H, *J* = 7.3 Hz), 4.17 (t, 2H, *J* = 7.3 Hz), 6.93 (s, 1H), 8.76 (s, 1H). ¹³C-NMR (100 MHz, DMSO-*d*₆): δ 13.9, 21.8, 27.6, 27.8, 53.9, 94.8, 99.3, 133.2, 143.1, 148.7, 153.8, 164.3. MS (ESI): *m/z* = 328.030, calcd. for C₁₂H₁₅N₃O₃Br *m/z* (M+H)⁺ = 328.029.

2.3.3. Synthesis of 4 (XLP4)—Under Argon atmosphere, a stirring solution of carboxylic acid **3** (0.50 g, 1.5 mmol) in anhydrous DMF (10 mL) was added *N*, *N*-diisopropylethylamine (0.80 mL, 4.6 mmol). The stirring mixture was kept at 0 °C for 10 min before HBTU (0.87 g, 2.3 mmol) was introduced. The solution was stirred for an additional 2 h at room temperature followed by addition of 1-adamantylamine (0.35 g, 2.3 mmol). The resulting mixture was stirred for 18 h and the solvent was subsequently evaporated under reduced pressure. The residue was dissolved in ethyl acetate (50 mL) and washed with saturated sodium bicarbonate aqueous solution (25 mL), water (25 mL) and brine (25 mL). The organic layer was dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography using ethyl acetate/hexanes = 1: 3 as the eluent to give the titled compound **4 (XLP4)** as a white solid (0.65 g, 93%); ¹H-NMR (400 MHz, CDCl₃, TMS): δ 0.92 (t, 3H, *J* = 6.9 Hz), 1.30-1.41 (m, 4H), 1.72 (br t, 6H, *J* = 14.5 Hz), 1.88 (quin, 2H, *J* = 7.3 Hz), 2.09-2.15 (br m, 9H), 3.97 (t, 2H, *J* = 7.4 Hz), 6.20 (s, 1H), 8.49 (s, 1H), 8.78 (br s, 1H). ¹³C-NMR (100 MHz, CDCl₃, TMS): 13.9, 22.3, 28.0, 28.6, 29.7, 36.6, 41.8, 52.4, 55.1, 93.4, 103.8, 134.5, 142.8, 145.3, 155.5, 161.2. MS (ESI): *m/z* = 461.146, calcd. for C₂₂H₃₀N₄O₂Br *m/z* (M+H)⁺ = 461.155.

2.3.4. Synthesis of 5—To a solution of bromo-pyrazolopyrimidine derivative **4** (50 mg, 0.11 mmol), 4-carboxyphenylboronic acid (40 mg, 0.24 mmol), potassium carbonate (44 mg, 0.33 mmol) in a mixture of 1,4-dioxane (1.5 mL) and water (0.5 mL) was added Pd(PPh₃)₄ (6.3 mg, 5.0 mmol). The resulting mixture was microwaved at 150 °C for 20 min in a sealed vessel and subsequently filtered through a pad of celite. The filtrate was

concentrated in reduced pressure. The residue was diluted in ethyl acetate (20 mL) and washed with 1% hydrochloric acid (20 mL). The aqueous layer was extracted with ethyl acetate (20 mL $\times$ 3). The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. The residue was purified by column chromatography using dichloromethane/methanol = 20 : 1 as the eluent to give the titled compound **5** as a white solid (44 mg, 81%); $^1\text{H-NMR}$ (400 MHz, CDCl_3 , TMS): δ 0.93 (t, 3H, J = 7.0 Hz), 1.25 (br s, 4H), 1.35-1.44 (m, 4H), 1.74 (br t, 6H, J = 14.5 Hz), 1.95 (quin, 2H, J = 7.3 Hz), 2.10-2.16 (br m, 9H), 4.09 (t, 2H, J = 7.2 Hz), 6.53(s, 1H), 8.15 (ABq, 4H, ν_{AB} = 20.0 Hz, J_{AB} = 8.6 Hz), 8.59 (s, 1H), 8.98 (br s, 1H). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3 , TMS): δ 13.8, 22.2, 28.0, 28.6, 29.5, 36.5, 41.7, 52.2, 87.8, 103.5, 126.8, 128.5, 130.2, 130.7, 136.5, 143.0, 145.4, 147.0, 156.7, 161.6. MS (ESI): m/z = 503.270, calcd. for $\text{C}_{29}\text{H}_{35}\text{N}_4\text{O}_4$ m/z ($\text{M}+\text{H}$) $^+$ = 503.265.

2.3.5. Synthesis of 6 (XLP6)—Under Argon atmosphere, a solution of benzoic acid derivative **5** (20 mg, 40 μmol) in anhydrous DMF (10 mL) was added *N*, *N*-diisopropylethylamine (21 μL , 0.12 mmol) and kept at 0 $^\circ\text{C}$ for 10 min before addition of HBTU (23 mg, 60 mmol). The mixture was stirred for 2 h at room temperature. The resulting solution was added into a solution of 1,6-diaminohexane (46 mg, 0.40 mmol) in anhydrous DMF (20 mL) over 15 min. The mixture was continuously stirred for 18 h before the solvent was removed in reduced pressure. The residue was diluted with ethyl acetate (20 mL) and washed with water (10 mL) and brine (10 mL). The aqueous layer was extracted with ethyl acetate (10 mL $\times$ 4). The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. The residue was purified by column chromatography using a gradient eluent of dichloromethane/methanol/ammonia = 100 : 5 : 1 to 100 : 15 : 1 to give the titled compound **6 (XLP6)** as a yellow paste (16 mg, 67%); $^1\text{H-NMR}$ (400 MHz, CDCl_3 , TMS): δ 0.88 (t, 3H, J = 6.8 Hz), 1.22-1.43 (m, 8H), 1.54 (m, 2H), 1.60-1.78 (m, 8H), 1.86 (br m, 3H), 2.02-2.16 (br m, 8H), 2.92 (t, 2H, J = 6.5 Hz), 3.34 (q, 2H, J = 6.1 Hz), 4.04 (t, 2H, J = 7.3 Hz), 5.35 (br s, 2H), 6.51 (s, 1H), 7.39 (br s, 1H), 7.83 (ABq, 4H, ν_{AB} = 30.6 Hz, J_{AB} = 7.9 Hz), 8.38 (s, 1H), 8.80 (s, 1H). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3 , TMS): δ 13.8, 22.2, 25.9, 26.2, 28.0, 28.5, 29.1, 29.5, 29.7, 36.4, 39.9, 40.7, 41.7, 52.1, 55.0, 88.3, 103.0, 126.6, 127.6, 134.0, 135.4, 142.8, 145.4, 154.6, 156.68, 161.4, 167.5. MS (ESI): m/z = 601.385, calcd. for $\text{C}_{35}\text{H}_{49}\text{N}_6\text{O}_3$ m/z ($\text{M}+\text{H}$) $^+$ = 601.386.

2.3.6. Synthesis of NIR760-XLP6—The following reaction and purification should preferably be carried out in the absence of light. Under Argon atmosphere, a solution of purified **NIR760** dye (20 mg, 21 μmol) in anhydrous DMF (1.0 mL) was added *N*, *N*-diisopropylethylamine (11 μL , 39 mmol) and stirred at 0 $^\circ\text{C}$ for 10 min before the addition of HBTU (12 mg, 31 mmol) and HOBt (5.3 mg, 31 μmol). The mixture was stirred for 1 h at room temperature. The resulting solution was added a solution of amine derivative **6** (8.0 mg, 13 mmol) in anhydrous DMF (0.50 mL). The mixture was continuously stirred for 18 h before the solvent was removed in reduced pressure. The residue was purified by semi-preparative reverse phase HPLC, using gradient eluents from 60% A to 5% A over 28 min, hold at 5% A for additional 10 min, and finally return to 60% A in 5 min. The low boiling solvent of the collected fractions (retention time = 8.9 min) was removed using rotary evaporation at 30 $^\circ\text{C}$ and the remaining solution was lyophilized to give the titled compound

NIR760-XLP6 as a dark green powder (9.0 mg, 45%); $^1\text{H-NMR}$ (400 MHz, $\text{DMSO-}d_6$): δ 0.86 (t, 3H, $J = 6.5$ Hz), 1.09 (s, 12 H), 1.32 (m, 4H), 1.42 (m, 4H), 1.55-1.72 (m, 10H), 1.83 (quin, 2H, $J = 7.2$ Hz), 1.96 (m, 6H), 2.56 (t, 4H, $J = 7.0$ Hz), 2.72 (m, 4H), 3.28-3.43 (m, 5 H), 4.26 (m, 6H), 6.42 (d, 2H, $J = 13.8$ Hz), 7.04 (d, 2H, $J = 13.8$ Hz), 7.25-7.44 (m, 5H), 7.52-7.67 (m, 4H), 8.02 (ABq, 4H, $\nu_{\text{AB}} = 27.9$ Hz, $J_{\text{AB}} = 8.12$ Hz), 8.13 (d, 2H, $J = 7.8$ Hz), 8.61-8.74 (m, 3H), 8.86 (s, 1H). $^{13}\text{C-NMR}$ (100 MHz, $\text{DMSO-}d_6$): 13.8, 21.7, 23.2, 24.20, 24.23, 26.4, 26.5, 27.0, 27.6, 27.8, 28.9, 29.1, 29.4, 36.0, 41.3, 42.7, 47.9, 48.1, 50.9, 53.9, 89.1, 98.6, 101.0, 101.7, 110.0, 110.09, 110.12, 119.6, 126.0, 126.1, 127.4, 127.8, 128.3, 129.2, 129.6, 130.7, 131.5, 133.6, 133.9, 134.0, 135.1, 135.90, 135.93, 136.4, 136.8, 140.0, 142.2, 143.2, 144.82, 144.84, 144.86, 144.90, 146.4, 147.0, 149.2, 153.5, 156.4, 161.3, 165.2, 165.5, 171.3. MS (ESI): $m/z = 780.133$, calcd. for $\text{C}_{79}\text{H}_{100}\text{N}_8\text{O}_{17}\text{S}_4$ m/z ($\text{M} + 5\text{H} + \text{CH}_3\text{OH}$) $^{2+} = 780.304$.

2.4. Saturation binding assay of NIR760-XLP6

Intact cell saturation binding assay was carried out to determine the binding affinity to CB_2R . Briefly, DBT- CB_2 cells were seeded onto 96 well optical bottom black plates and incubated for 24 h. Cells were fixed with 4% paraformaldehyde in PBS for 10 min at 25 °C. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min at 25 °C. Next, cells were pre-incubated with blocking agent **XLP4** (10 μM) or vehicle for 30 min, and then incubated with an increasing concentration of **NIR760-XLP6** (5.0 nM to 0.90 μM) for 30 min. Cells were rinsed once with PBS to remove remaining probes in the medium. Fluorescence (excitation/emission = 740/790 nm) was recorded with a Synergy^{TM} H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). The specific binding was obtained by the subtraction of non-specific binding (with blocking agent) from total binding (without blocking agent). The dissociation constant (K_d) and receptor density (B_{max}) were estimated from the non-linear fitting of specific binding versus **NIR760-XLP6** concentration using Prism software (GraphPad, San Diego, CA). To determine that binding affinity of **NIR760-XLP6** to CB_1R , DBT- CB_1 cells were treated similarly using SR-141716A (1.0 mM) as the blocking agent and increasing concentration of **NIR760-XLP6** (10 μM to 0.20 mM).

2.5. Viability assay of NIR760-XLP6

The cell viability assay was performed using DBT- CB_2 cells. The cells were treated with various concentrations of **NIR760-XLP6** ranging from 0 to 50 μM , with indocyanine green (ICG) serving as the negative control and Doxorubicin as the positive control. Subsequently, the cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2 for 24 h. After the incubation, cell viability was assessed by the CellTiter-Glo® Luminescent Cell Viability Assay kit according to the manufacturer supplied standard protocols. The CellTiter-Glo® Luminescent Cell Viability assay measures the number of viable cells based on the quantitation of the ATP present. The luminescence signal was detected at 528 nm.

2.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

DBT-CB₂ or DBT-CB₁ cells were seeded onto 3.5 mm dishes and cultured at 37 °C for 24 h. Total RNA was extracted from DBT-CB₂ or DBT-CB₁ cells using RNeasy Lysis Reagent. First-strand cDNA was synthesized from total RNA using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). For RT-PCR assays, the sequences of primers used for CB₂R were 5'-tctatcattacgcctgc-3' (sense) and 5'-ggctcctagtggtttcacatcagcctc-3' (antisense); for CB₁R were 5'-cacaagcagccaataacaca-3' (sense) and 5'-acagtgccttgatgcagcttc-3' (antisense); and for GAPDH were 5'-tgaacgggaagctcactggcat-3' (sense) and 5'-tgcctgcttcaccactcttg-3' (antisense). Amplifications were run using Platinum Taq DNA polymerase and consisted of 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C.

2.7. Cell fluorescent imaging of NIR760-XLP6

DBT-CB₂ or DBT-CB₁ cells were seeded into 8-well chamber slide. Four groups of samples are prepared as follows: (1) DBT-CB₂ cells were incubated with 5.0 µM of **NIR760-XLP6** at 37 °C for 30 min; (2) DBT-CB₁ cells were incubated with 5.0 µM of **NIR760-XLP6** at 37 °C for 30 min; (3) DBT-CB₂ cells were incubated with 10 µM of **XLP4** at 37 °C for 30 min, followed by 5.0 µM of **NIR760-XLP6** at 37 °C for 30 min; and (4) DBT-CB₂ cells were incubated with 5.0 µM of **NIR760** at 37 °C for 30 min. After the incubation, cells were washed three times with PBS, and fixed with 4% paraformaldehyde in PBS for 20 min at 25 °C. Cells were then permeabilized with 0.1% Triton X-100 in PBS. The cell nucleus was stained with 1.0 µg/mL DAPI for 15 min at 25 °C. Cells were mounted with ProLong® Gold antifade reagent and then imaged using the Zeiss Axio Observer fluorescent microscope equipped with the ApoTome 2 imaging system (Carl Zeiss Microimaging GmbH, Jena, Germany). Fluorescence images were captured with an NIR-sensitive camera with an ICG filter (excitation/emission: 750-800/820-875 nm). Nuclear images were obtained with a DAPI filter (excitation/emission: 335-383/420-470 nm). Differential interference contrast (DIC) images were obtained through Trans light DIC.

2.8. Quantitative binding assay of NIR760-XLP6

1×10^5 (per well) DBT-CB₂ or DBT-CB₁ cells were seeded onto 96-well optical black plates. The cells were subsequently incubated at 37 °C under a humidified atmosphere of 5% CO₂ for 24 h. Cells were divided into four groups: (1) DBT-CB₂ cells incubated with 5.0 µM of **NIR760-XLP6** at 37 °C for 30 min; (2) DBT-CB₁ cells incubated with 5.0 µM of **NIR760-XLP6** at 37 °C for 30 min; (3) DBT-CB₂ cells incubated with 10 µM of **XLP4** at 37 °C for 30 min, followed by 5.0 µM of **NIR760-XLP6** at 37 °C for 30 min; and (4) DBT-CB₂ cells incubated with 5.0 µM of **NIR760** at 37 °C for 30 min. A Synergy™ H4 Hybrid Multi-Mode Microplate Reader was used to read fluorescence at 740/790 nm (excitation/emission). After the initial reading, cells were rinsed with cell culture medium twice and the fluorescence emission was measured again. The fluorescence emissions recorded were normalized by dividing each point by the initial reading and then corrected with cell number (with data expressed in terms of relative fluorescence units, RFU). The cell numbers were obtained as follow: cells were fixed in 4% paraformaldehyde in PBS for 10 min at 25 °C, permeabilized with 0.1% Triton X-100 in PBS for 5 min at 25 °C and rinsed twice with

PBS. DRAQ-5 (1 : 2500) was then added at 25 °C and cells were rinsed three times with PBS after 5 min. Fluorescence at 650/690 nm (excitation/emission) was recorded for the comparison of cell number.

2.9. In vivo optical imaging of NIR760-XLP6

A total of twelve mice were divided into four groups. Subcutaneous tumors were developed by injecting 1×10^6 DBT-CB₂ cells ($n = 9$) or 1×10^6 DBT-CB₁ cells ($n = 3$) into the right flank of 6 - 8 week old female nu/nu mice. Experiments with tumor-bearing mice were performed 10 days after the injection of tumor cells. Nude mice were anesthetized with a 2.5% isoflurane/oxygen gas mixture. All mice were injected with imaging agents in 100 μ L saline via tail vein as follows: (1) three DBT-CB₂ tumor bearing mice with 10 nmol of **NIR760-XLP6**; (2) three DBT-CB₁ tumor bearing mice with 10 nmol of **NIR760-XLP6**; (3) three DBT-CB₂ tumor bearing mice with 100 nmol of **XLP4** followed by 10 nmol **NIR760-XLP6** after 1 h; (4) three DBT-CB₂ tumor bearing mice with 10 nmol of **NIR760**.

Optical imaging was performed with a charge-coupled device camera-based bioluminescence imaging system IVIS Lumina XR (PerkinElmer, Waltham, MA) using the following parameters: excitation filter, 745 nm; emission filter, 800 nm; exposure time, 1 sec; binning, small; field of view, 12; f /stop, 2; open filter. The images were captured at pre-injection, 1 h, 3 h, 6 h, 9 h, 24 h, 48 h, and 72 h post-injection. Signal was displayed as Radiant Efficiency ([photons/sec/cm²/sr]/[μ W/cm²]). Images were analyzed by using Living Image 4.4 software (Caliper Life Sciences, Hopkinton, MA). For the data analysis of tumor contrast, region-of-interest (ROI) of the tumor site at the right flank of the animal (T) and normal tissue at the left flank (N) were drawn. Tumor contrast enhancement (T/N) profiles were obtained by dividing fluorescence intensity at T by that at N as shown in Table SI-2.

2.10. Ex vivo imaging and biodistribution

After the last time point of in vivo optical imaging, all mice were euthanized. Tumors and selected organs (blood, heart, lung, liver, spleen, pancreas, kidneys, muscle from left leg, and brain) were excised and imaged under IVIS Lumina XR system.

The biodistribution of **NIR760-XLP6** and **NIR760** in the corresponding mice groups was evaluated by drawing ROI along the excised tumors and organs. Fluorescence signal ratios of target (tumor or organ) over normal (muscle) are shown in Table SI-3.

2.11. Data Processing and Statistics

All of the data given are the mean $\pm$ SEM (standard error of the mean) of n independent measurements. For in vitro binding study and ex vivo biodistribution study, statistical analyses were performed using one-way ANOVA method, with p values <0.05 considered statistically significant. For in vivo imaging studies, statistical analyses were performed using repeated measures ANOVA (all data from 0 – 72 h were utilized), with p values <0.05 considered statistically significant.

3. Results and Discussion

3.1. Design and synthesis of NIR760-XLP6

Scheme 1 depicts the synthetic pathway of **NIR760-XLP6**, which consists of a NIR fluorescent dye **NIR760** and a new functional CB₂R targeting ligand termed as **XLP6**. We recently reported **NIR760** as a highly fluorescent dye with absorption and emission close to 800 nm, the most optimal wavelength for deep tissue imaging.[21] The ligand **XLP6** was designed based on the pyrazolo[1,5- α]pyrimidine structure, which was recently reported to have high CB₂R binding affinity and selectivity.[29] A unique feature of pyrazolopyrimidine molecules is that chemical modification of the moieties at the C2 position does not change their binding affinity and specificity at CB₂R to a great extent. [29] Such an observation is rare in structure-activity relationship (SAR) studies of CB₂R ligands and provides a unique opportunity for introducing functional groups at the desired strategic position. This is supported by the molecular modeling study, which indicates that the *para*-position of the phenyl ring may be exposed to an empty space of the binding pocket when the molecule binds to CB₂R. To synthesize the functional analog of the pyrazolopyrimidine molecules, we started from 3-bromo-5-aminopyrazole using the reported procedure.[27] The bromo group at C-2 position not only eliminates the potential selectivity challenges between two carboxylic acids during amide coupling with 1-adamantylamine but also allows easy transformation to *para*-benzoic acid moiety using Suzuki coupling. Next, we introduced terminal amino group to **XLP6**, which allows for universal coupling to signaling moieties.

To synthesize **NIR760-XLP6**, pyrazolo[1,5- α]pyrimidine derivative **1** was prepared from 3-bromo-5-aminopyrazole according to literature.[27] Next, the secondary amine was successfully alkylated with 1-bromopentane to give compound **2**. Carboxylic acid derivative **3** was obtained through a base hydrolysis of **2** and was subsequently coupled with 1-adamantylamine to produce the CB₂R recognizing ligand as compound **4** (**XLP4**). A microwave assisted Suzuki coupling[30] between **4** and 4-carboxyphenylboronic acid using Pd(PPh₃)₄ as the catalyst provided benzoic acid derivative **5**. Next, a hexanediamine linker was introduced via amide coupling to give ligand **XLP6**. Finally, another amide coupling reaction between **XLP6** and **NIR760** (synthesized according to our previous report[21]) yielded **NIR760-XLP6**. The structures were fully characterized by ¹H NMR, ¹³C NMR and MS analysis (Figure SI-1 and Figure SI-2 in the Supporting Information)

3.2. Photophysical properties of NIR760-XLP6

The absorption and emission spectra of **NIR760-XLP6** are shown in Figure 1 and compared with those of **NIR760**. The attachment of **XLP6** to **NIR760** resulted in a redshift of the maximum absorption from 760 to 766 nm. The molar extinction coefficient of **NIR760-XLP6** is similar to that of **NIR760** at 2.0×10^5 and 2.1×10^5 M⁻¹cm⁻¹, respectively. The fluorescence emission of **NIR760-XLP6** also exhibits a redshift to 785 nm with a quantum yield of 11.6% in water, as compared to **NIR760** at 780 nm with a quantum yield of 12.0%. (Table SI-1 in the Supporting Information).[31]

3.3. Saturation binding study of NIR760-XLP6

The binding affinity of **NIR760-XLP6** with DBT-CB₂ cells was tested at endogenous levels.[32] To saturate receptor-binding sites and account for non-specific binding, a large excess of **XLP4** was added to a parallel set of DBT-CB₂ cells. We found that **NIR760-XLP6** bound to CB₂R with a nanomolar affinity ($K_d = 169.1 \pm 66.09$ nM) (Figure SI-3 in the Supporting Information). In contrast, **NIR760-XLP6** does not bind to DBT-CB₁ cells, as the binding affinity was incalculable ($K_d > 10000$ nM, a CB₁R ligand, SR141716A, was used as the blocking agent).

During the study, noticeable amount of non-specific binding of NIR760-XLP6 was observed. Unfortunately, this is a common challenge in the development of CB₂R fluorescent probes. Introduction of a bulky fluorophore often leads to compromised ligand binding property.[17] Possible improvements of CB₂R fluorescent probes design to address this issue include using smaller fluorescent dyes or developing advanced probe development strategies such as bioorthogonal chemistry. Cytotoxicity study of **NIR760-XLP6**

To examine the cytotoxicity of **NIR760-XLP6**, we used Doxorubicin, a commonly used chemotherapy drug, as the positive control and ICG, the only FDA approved NIR fluorescent dye, as the negative control. **NIR760-XLP6** showed comparable low cytotoxicity with ICG whereas Doxorubicin showed high cytotoxicity at high concentrations (Figure SI-4 in the Supporting Information).

3.4. RT-PCR

The cannabinoid receptor expression in DBT-CB₂ and DBT-CB₁ cells were confirmed using RT-PCR. As expected, CB₂R positive band at 165 bp and CB₁R positive band at 65 bp were detected in DBT-CB₂ and DBT-CB₁ cells respectively (Figure SI-5 in the Supporting Information).

3.5. In vitro binding studies of NIR760-XLP6

We next studied the selectivity and specificity of **NIR760-XLP6** with in vitro fluorescence imaging using DBT-CB₂ and DBT-CB₁ cells (see Figure 2a).[32] The two DBT cells were treated with **NIR760-XLP6** and imaged using NIR fluorescence microscopy. We found that DBT-CB₂ cells showed remarkably higher fluorescence signal than DBT-CB₁ cells, indicating preferable binding of **NIR760-XLP6** to CB₂R over CB₁R. Next, DBT-CB₂ cells were pretreated with **XLP4** as the blocking agent followed by **NIR760-XLP6** treatment for specificity study. Compared to unchallenged cells, **XLP4** significantly reduced the uptake of **NIR760-XLP6** in DBT-CB₂ cells. Furthermore, DBT-CB₂ cells treated with non-targeted **NIR760** failed to show significant fluorescence signal. These data suggest specific binding of **NIR760-XLP6** to the target CB₂R.

To corroborate these finds, as well as quantitatively evaluate the selectivity and specificity of **NIR760-XLP6** binding towards CB₂R, we carried out intact cell uptake study using a multi-mode microplate reader as the second independent method. The results are shown in Figure 2b (in relative fluorescence units, RFU). **NIR760-XLP6** treated DBT-CB₂ cells showed approximately 40% higher fluorescence signal than that of DBT-CB₁ cells (RFU:

176.3 $\pm$ 17.2 vs 103.5 $\pm$ 4.2, ** p < 0.01, respectively). Blocking agent **XLP4** effectively reduced the fluorescence signal of **NIR760-XLP6** in DBT-CB₂ cells (RFU: 93.7 $\pm$ 15.2 vs 176.3 $\pm$ 17.2, ** p < 0.01, respectively). In addition, significantly lower fluorescent signal was observed when the DBT-CB₂ cells were treated with non-targeted **NIR760** dye instead of **NIR760-XLP6** (RFU: 25.8 $\pm$ 3.4 vs 176.3 $\pm$ 17.2, *** p < 0.001, respectively). These results indicate that **NIR760-XLP6** binds to CB₂R both preferably and specifically.

3.6. In vivo optical imaging studies of NIR760-XLP6

To test the preferable targeting of **NIR760-XLP6** to CB₂R over CB₁R in vivo, we imaged the probe uptake in tumor mouse model system. In vivo imaging experiments commenced approximately 10 days post-inoculation of DBT-CB₂ and DBT-CB₁ cells subcutaneously into the right flank of 6 - 8 week old female nu/nu mice. A total of twelve mice was divided into four groups to determine the time-resolved accumulation (n = 3 for each group): (1) DBT-CB₂ tumor bearing mice injected with **NIR760-XLP6** (10 nmol); (2) DBT-CB₁ tumor bearing mice injected with **NIR760-XLP6** (10 nmol); (3) DBT-CB₂ tumor bearing mice injected with **XLP4** (100 nmol) followed by **NIR760-XLP6** (10 nmol) after 1 h; and (4) DBT-CB₂ tumor bearing mice injected with free dye **NIR760** (10 nmol). The resulting sizes and overall tumor growth of each set of DBT tumors showed no significant difference (data not shown). For time-resolved accumulation, images were captured at pre-injection, 1, 3, 6, 9, 24, 48, and 72 h post injection. One representative mouse from each group is shown and all images are time-dependent fluorescence images overlaid on the corresponding white light images (Figure 3a). To quantitatively compare the imaging contrast in the tumor region among the four groups of animals, region-of-interests (ROIs) at the tumor and normal tissue area in the left flank were selected. The fluorescence signal intensity of the tumor area (T) was normalized to that of the normal area (N) and plotted against time points to evaluate time-dependent tumor contrast enhancement (T/N) profiles (Figure 3b and Table SI-2 in the Supporting Information).

Upon injection, **NIR760-XLP6** dispersed rapidly in mice during the first 9 h and then underwent a delayed clearance after 24 h. Higher T/N ratios were observed in DBT-CB₂ tumor group than in the DBT-CB₁ tumor group at 48 h (1.6 $\pm$ 0.0 vs 1.3 $\pm$ 0.1, *** p < 0.001, respectively) and 72 h (2.0 $\pm$ 0.1 vs 1.3 $\pm$ 0.1, **** p < 0.0001, respectively) post-injection. In compare with the **NIR760-XLP6** treatment only DBT-CB₂ tumor group, pretreatment of **XLP4** reduced the T/N ratio at 48 h (1.6 $\pm$ 0.0 vs 1.4 $\pm$ 0.1, * p < 0.05, respectively) and 72 h (2.0 $\pm$ 0.1 vs 1.5 $\pm$ 0.1, **** p < 0.0001, respectively) post-injection. In contrast, **NIR760** exhibited rapid biodistribution within 1 h post-injection followed by quick tissue clearance afterwards. The T/N ratio of free dye **NIR760** was lower than that of **NIR760-XLP6** at 48 h (1.4 $\pm$ 0.1 vs 1.6 $\pm$ 0.0, ** p < 0.01, respectively) and 72 h (1.3 $\pm$ 0.1 vs 2.0 $\pm$ 0.1, **** p < 0.0001, respectively) post-injection. It is noteworthy that, after 6 h post-injection, the overall fluorescence intensity observed in this free dye group was more than one order of magnitude lower than that of the other groups (Figure SI-6 in the Supporting Information).

3.7. Ex vivo imaging and biodistribution study

To study the biodistribution and further evaluate the binding selectivity of **NIR760-XLP6**, all mice were euthanized after the last imaging time point. Tumor and selected organs

(blood, heart, lung, liver, spleen, pancreas, kidneys, muscle from left leg, and brain) were excised and imaged (see Figure 4a). To assess the uptake of the **NIR760-XLP6**, ROIs along the excised tumors and organs were drawn and the corresponding fluorescence intensities were normalized to that of leg muscle. The target/normal signal ratio data were summarized in Figure 4b. Tumor contrast as high as 7.9 was observed in the DBT-CB₂ tumor mice injected with **NIR760-XLP6**, which is 2-fold higher than that in the DBT-CB₁ tumor mice (7.9 ± 0.8 vs 3.8 ± 0.7 , $**p < 0.01$, respectively). Besides, certain organs, including lung, liver and kidney, also showed high target/normal ratios. However, no significant difference of target/normal ratios between DBT-CB₂ and DBT-CB₁ tumor mice was observed in these organs (Figure 4b and Table SI-3 of the Supporting Information). Furthermore, the DBT-CB₂ tumor mice with **XLP4** pretreatment revealed lower tumor contrast than that of non-blocking group (5.6 ± 0.7 vs 7.9 ± 0.8 , $*p < 0.05$, respectively). Additionally, the **NIR760** group showed significantly lower tumor contrast than **NIR760-XLP6** injected DBT-CB₂ tumor group (1.8 ± 0.4 vs 7.9 ± 0.8 , $***p < 0.001$, respectively). These results are consistent with those from in vitro and in vivo imaging studies, and provide further evidence on the preferable and specific binding of **NIR760-XLP6** to CB₂R over CB₁R.

4. Conclusion

In conclusion, we successfully developed a novel NIR fluorescent molecular probe, **NIR760-XLP6**, which preferentially binds to CB₂R over CB₁R both in vitro and in vivo. To the best of our knowledge, **NIR760-XLP6** is the first optical imaging tool suitable for visualization of CB₂R over CB₁R preferentially. We envision that such a molecular probe will have great potential in studying the role of CB₂R in various diseases and physiological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Normalized UV-vis absorption (solid lines) and emission (dotted lines) spectra of **NIR760-XLP6** (green) and **NIR760** (red) in water at a concentration of 1×10^{-6} M ($\lambda_{\text{ex}} = 725$ nm).

Figure 2. **NIR760-XLP6** preferentially and specifically binds to CB₂R over CB₁R. (a) Fluorescence microscopy imaging of DBT-CB₂ and DBT-CB₁ cells. From left to right: NIR fluorescence (ICG filter, red), nuclear staining (DAPI filter, blue), differential interference contrast (DIC). Scale bar: 20 μ m. (b) Cellular uptake of fluorescent probes is represented as relative fluorescence units (RFU) as quantified using a multiplate reader. Cells were divided into four groups as follow: (1) DBT-CB₂ cells treated with **NIR760-XLP6**, (2) DBT-CB₁ cells treated with **NIR760-XLP6**, (3) DBT-CB₂ cells treated with **XLP4** followed by **NIR760-XLP6**, and (4) DBT-CB₂ cells treated with **NIR760**. Each data point represents the mean $\pm$ SEM based on triplicate samples.

Figure 3.

In vivo tumor optical imaging of **NIR760-XLP6**. (a) Mice images, at pre-injection, 1h, 3 h, 6 h, 9 h, 24 h, 48 h, and 72 h post-injection. All images are displayed at the same scale. (b) Tumor contrast enhancement (T/N signal ratio) profiles. All mice were divided into four groups as follow: (1, red ●) three DBT-CB₂ tumor-bearing mice injected with **NIR760-XLP6**; (2, green ■) three DBT-CB₁ tumor-bearing mice injected with **NIR760-XLP6**; (3, blue ▲) three DBT-CB₂ tumor-bearing mice injected with **XLP4** followed by **NIR760-XLP6**; and (4, black ▼) three DBT-CB₂ tumor-bearing mice injected with **NIR760**. Each data point represents the mean $\pm$ SEM based on triplicate samples.

Figure 4.

Ex vivo optical imaging and biodistribution study of **NIR760-XLP6**. (a) Ex vivo imaging of excised tumor and organs at 72 h post-injection from DBT-CB₂ or DBT-CB₁ tumor-bearing mice. The white arrow points to the tumor. (b) Graphical quantification of target/normal signal ratio. All mice were divided into four groups as follow: (1, red) Three DBT-CB₂ tumor-bearing mice injected with **NIR760-XLP6**; (2, green) Three DBT-CB₁ tumor-bearing mice injected with **NIR760-XLP6**; (3, blue) Three DBT-CB₂ tumor-bearing mice injected with **XLP4** followed by **NIR760-XLP6**; and (4, black) Three DBT-CB₂ tumor-bearing mice injected with **NIR760**. Each data point represents the mean $\pm$ SEM based on triplicate samples.

Scheme 1. Synthesis of NIR760-XLP6

Reagents and conditions: (a) Diethyl ethoxymethylenemalonate, acetic acid, 120 °C, 3 h. (b) 1-Bromopentane, K_2CO_3 , DMF, 100 °C, 23 h. (c) 10% NaOH(aq), MeOH, 25 °C, 1 h, then pH = 4. (d) 1-Adamantylamine, DIEA, HBTU, DMF, 25 °C, 18 h. (e) 4-Carboxyphenylboronic acid, K_2CO_3 , Pd(PPh₃)₄, 1,4-dioxane, water, microwave, 20 min. (f) DIEA, HBTU, DMF, 2 h, 0 °C, then 1,6-diaminohexane, 25 °C, 18 h. (g) NIR760, DIEA, HBTU, HOBt, DMF, 25 °C, 18 h.